THE ROLE OF METASTASIS INDUCING PROTEINS IN ENDOMETRIAL CANCER

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ABSTRACT

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INTRODUCTION

Prognosis of women who develop advanced endometrial cancer is poor, with a 5 year survival rate of only 45% and 25% for stages III and IV respectively. Distant metastasis of malignant endometrial cells beyond the primary focus contributes to the advanced disease stages. A group of proteins, known as metastasis-inducing proteins (MIPs), have been shown to induce cellular invasion and metastasis, specifically AGR2, S100A4, S100P and Osteopontin (OPN).

METHODS

To investigate the expression of these four MIPs in endometrial cancer, and compare it to the endometrium of fertile control and postmenopausal women. Endometrial samples were collected from 55 women; 30 with endometrial cancer, 15 normal fertile control endometrium in the proliferative phase of the cycle and 10 postmenopausal. The expression of MIPs was evaluated by immunohistochemistry and confirmatory testing was undertaken with reverse transcriptase PCR.

RESULTS

All endometrial cancer samples showed a significantly increased immuno-reactivity to S100P (Stromal, $p = 0.0221$) and S100A4 (Stromal, $p = 0.0275$) compared to benign endometrium. However OPN was virtually absent (Glands $p = 0.0047$, luminal

epithelium p=0.0018) in endometrial cancer cells. All postmenopausal samples showed a significantly decreased immuno-reactivity to all the MIPs (OPN, $p = 0.0001$, S100P, $p = 0.0002$, AGR2, $p > 0.0001$, S100A4, $p = 0.0004$) compared to endometrial cancer cells. RT-PCR results confirmed IHC results for the expression of S100P, AGR2 and S100A4, but contradicted OPN IHC results. The RT-PCR results gained were not statistically significant.

DISCUSSION

This data provides an interesting insight into the metastatic process of endometrial cancer. My results suggest that all four MIPs play a key role in metastasis of endometrial cancer, in comparison to the normal postmenopausal endometrium. Further research is required to understand the extent of the involvement OPN, S100P, AGR2 and S100A4 in inducing the metastatic process.

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LIST OF ABBREVIATIONS

EEC – Endometrioid endometrial carcinoma

NEEC – Non- endometrioid endometrial carcinoma

MMMT – Malignant mixed mullerian tumour

FIGO - International federation of gynecology and obstetrics

MIPS - Metastasis inducing proteins

OPN – Osteopontin

AGR2 – Anterior gradient homolog 2

HEEC - Human endometrial endothelial cells

siRNA - Short interference RNA

ISK - Ishikawa

RAGE - Receptor for advanced glycation end products

MAP - mitogen-activated protein

NFkB - nuclear factor-B

SOP – Standard operating protocol

NBF - Neutral buffered formalin

PBS - Phosphate buffered saline

TBS – Tris buffered saline

EDTA - Ethylenediaminetetraacetic acid

IHC - Immunohistochemisty

HIER - Heat induced epitope retrieval

HRP - Horseradish peroxidase

DAB - Diaminobenzidine

RT-PCR - Reverse transcription - polymerase chain reaction

PCR - Polymerase chain reaction

cDNA - Complementary DNA

mRNA – Messenger RNA

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1 INTRODUCTION

The human endometrium plays a dynamic role in ensuring uterine cavity patency, implantation and undergoes cyclical regeneration. Menstruation is cyclically synchronized to the excretion of hormones by the ovaries. Towards the end of the female reproductive period the depletion of oocytes and reduction in levels of oestrogen and progesterone leads to the cessation of menstruation. This is otherwise known as the menopause. Following this period (post-menopause), the endometrium can be exposed to high levels of unopposed oestrogen (with the absence of progesterone), which increases the risk of endometrial proliferation, hyperplasia and carcinoma. The distant spread of advanced cancerous endometrial cells beyond the primary focus, otherwise known as metastasis, is associated with increased mortality. The metastatic process is poorly understood and extensive research is required. It has been postulated that cellular processes such as increased mobility, proliferation, survival of cancerous cells etc may play an important role in allowing cells to adhere and continue growing in a hostile environment. Some researchers suggest that there are specific types of cells, chemokines, proteins, enzymes, or immune cells within the cancer that can have the ability to initiate the metastasis process. We aim to further understand the metastatic process in endometrial cancer, by endeavoring to identify cancerous cells that have a tendency to metastasise. This would provide therapeutic targets for new treatment, improving prognosis and reducing mortality rates.

1.1 THE ENDOMETRIUM

The uterine cavity is a single camber organ comprised of (from outside): the peritoneum which consists of the broad and suspensory ligaments; the peritetrium a thin serosal lining, the myometrium, a fibromuscular wall and finally the innermost two-layered modified mucosa, the endometrium (Edmonds & Dewhurst 2007; Critchley & Saunders 2009).

The uterine artery supplies blood to the uterus, which is derived from the anterior division of the internal iliac artery. The uterine artery anastomoses with the ovarian artery creating arcuate branches supplying the myometrium and endometrium via the spiral arteries (Edmonds & Dewhurst 2007; Mutter & Ferenczy 2001).

The endometrium is a diverse and dynamic tissue primarily preparing for implantation and functioning as the decidua during pregnancy. If pregnancy is not established, the endometrium continues to undergo well-organised cycles of menstruation, proliferation differentiation and regeneration (Jabbour et al 2006; Gregory et al 2002)

The endometrium can be identified as having two layers, the functional layer (stratum functionalis) and the basal layer (stratum basalis). The functionalis represents the upper layer of the endometrium and consists of a superficial layer with a few simple tubular glands set in an abundant vascularised stroma (zona compacta) and a deeper layer with increased number of glands, but relatively less stroma (zona spongiosa). This layer is shed once a month during menstruation and is regenerated from the underlying basalis, which projects into the myometrium (Jimenez-Ayala & Jimenez-Ayala Portillo 2008; Jabbour et al 2006). The activity and structure of the functionalis

is highly responsive to the hormones cyclically produced by the ovaries, in comparison to the basalis.

The menstrual cycle is a highly regulated sequence of events, which involves an intimate relationship between the hypothalamus, pituitary, ovaries and uterus. It is predominantly synchronized with the ovarian cycle (Edmonds & Dewhurst 2007; Critchley & Saunders 2009). Ovarian steroids, oestrogen and progesterone play a crucial role in regulating the changes seen in the endometrial structure during the menstrual cycle. Following the oestrogen primed endometrium, progesterone is essential for the establishment and maintenance of pregnancy (Jabbour et al 2006).

There are three key phases of the 28-30 day menstrual cycle: The menstrual phase, in which the endometrium is shed following reduced levels of progesterone, in response to a failed implantation. The oestrogen driven proliferative phase, where the endometrium rapidly regenerates and proliferates, follows this and is also well synchronised with the growth and maturation of an oocyte and its follicle. The secretory phase, in conjunction with the final maturation and release of the oocyte, is dominated by progesterone resulting in endometrial differentiation (Jabbour et al 2006; Edmonds & Dewhurst 2007}.

Towards the end of the reproductive life span women go through menopause, at the average age of 51 years (Edmonds & Dewhurst 2007). Menopause is defined as the last menstrual period, from the Greek “Menos” meaning month and “Pausis” meaning cessation. It can only be diagnosed retrospectively after a minimum one year of amenorrhoea. The reduction and ultimate depletion of oocytes is the key in the cessation of menstruation. Menopause is also associated with a permanent cessation

of progesterone production and reduced levels of oestrogen available to the endometrium.

Following both the physiological decline in ovarian function and reduced levels of ovarian hormone levels, over a few years the non-functioning endometrium progressively changes to an atrophic endometrium. Atrophic endometrium is very thin, consisting of narrow glands lined by a low epithelium set in a dense fibrous stroma of spindle cells. It is near impossible to distinguish the two layers of the endometrium, functionalis and basalis, in an atrophic endometrium (Jimenez-Ayala & Jimenez-Ayala Portillo 2008; Nalaboff et al 2001).

The life expectancy of women is steadily rising, and is currently at 82 years of age in the UK, hence increasing the time spent living with menopause and its effects (Edmonds & Dewhurst 2007). Quality of life can be severely affected in women in their post-menopausal phase. Early menopausal effects include vasomotor and psychological changes, and more long term is increased risk in developing osteoporosis, cardiovascular problems and dementia (Edmonds & Dewhurst 2007).

Some post-menopausal women are exposed to different levels of unopposed oestrogen, i.e. in the absence of progesterone. This continuing exposure can lead to abnormal endometrial proliferation, hyperplasia and in extreme cases carcinoma (Edmonds & Dewhurst 2007). Excess unopposed oestrogen is a risk factor for endometrial hyperplasia that can predispose to endometrial carcinoma, along with obesity and null parity. With the epidemic of obesity and rising life expectancy there is no surprise the incidence of endometrial hyperplasia and cancer is rising.

1.2 ENDOMETRIAL CANCER

Cancer of the lining of the uterus predominately affects post-menopausal women aged between 55 and 70 years, with a few cases occurring before age 40. The incidence of endometrial cancer has been reported to be 44 in 100,000 and is constantly rising as life expectancy increases (Executive, NHS 1999; Jemal et al 2009). It was the cause of 1,741 deaths in the UK in 2008 (Cancer research UK). The rise continues to place a financial burden on the NHS. Annual costs for gynaecological cancer surgeries are estimated to double on average (average rise of almost £200,00). Increased endometrial cancer referral to Cancer Centers are estimates to increase annual costs for surgery by about 90% (range: £0 to £120,00). (Executive, NHS 1999).

The most important risk factors for endometrial cancer are postmenopausal status, body mass index >25kg/m^2, hyperoestrogenism (for example, nulliparity/infertility, anovulation, late onset menopause) and unopposed exogenous oestrogen use, for example tamoxifen. In premenopausal women, obesity can cause insulin resistance, ovarian androgen excess, anovulation, and chronic progesterone deficiency (Masciullo et al 2010). In postmenopausal women, the conversion of androgens to oestrogen is enhanced in peripheral fat stores (Masciullo et al 2010). Considering the challenges we face in tackling obesity in our population, it though to be an increasingly common risk factor responsible for the development of endometrial cancer.

Half of patients diagnosed with endometrial cancer have identifiable risk factors, while the other half appear to be at low risk (Masciullo et al 2010, Amant et al 2005).

This highlights the importance of early detection and surveillance. The primary presenting symptom patients develop is an abnormal bleeding pattern, either post-menopausal or inter-menstrual bleeding. This can allow for early detection of the cancer, and by laying a good management plan, improve the survival rates. Although the 5-year survival is good for stage I & II, it is only 45% and 25% for stages III & IV, respectively (Abeler & Kjorstad 1991; Morrow et al 1991; Grigsby et al 1992). The survival rates for advanced endometrial cancer are worse than other common gynaecological cancers, such as ovarian cancer.

Bokhman (1983) broadly categorised endometrial cancer into two groups: estrogen-dependent endometrioid endometrial carcinomas (EECs), or Type 1, and non-endometrioid endometrial carcinomas (NEECs), or Type 2 tumours. These groups can be further sub-divided with variants of Type 1 endometrioid adenocarcinoma (See Table 1). Cancers are grouped depending on histopathology, epidemiology, and clinical behaviour. (Saso et al 2011; Okuda et al 2010).

Table 1: Endometrial cancer types

Type 1	**Type 2**
Endometrioid endometrial adenocarcinoma: - With squamous differentiation - Villoglandular - Secretory - With ciliated cells - Mucinous carcinoma	**Non-endometrioid carcinoma:** - Serous carcinoma - Clear-cell carcinoma - Mixed carcinoma - Squamous-cell carcinoma - Transitional- cell carcinoma - Small-cell carcinoma - Undifferentiated carcinoma.

Approximately 70-80% of diagnosed endometrial carcinomas are classified as EEC Type 1 (Okuda et al 2010). EECs arise from atypical complex hyperplasia and are largely oestrogen driven, which exhibit low histological grade and therefore a favourable prognosis. Since the oestrogen responsiveness of the endometrial cells are mediated via oestrogen receptors and more differentiated cells possess steroid receptors, it is believed that more differentiated cancers are hormone responsive (hence less aggressive). The rare mucinous carcinomas are also considered Type 1 carcinomas because they are of low histological grade and usually express oestrogen and/or progesterone receptors (Samarnthai et al 2010).

On the other hand prognosis is poor with Type 2 NEECs, which are rare, occurring in approximately 20-30% of all women diagnosed with endometrial cancer (Okuda et al 2010). Women with Type 2 endometrial cancer, predominately high grade serous and clear-cell carcinomas are not related to the oestrogen pathway and arise in the environment of an atrophic endometrium (Prat et al 2007; Okuda et al 2010; Potischman et al 1996). They are at a higher risk of relapse and they develop metastatic disease due to the aggressive nature of the cancer (Amant et al 2005).

It is important to note that grouping of cancers is not strict, as there is an increasing incident of endometrial cancers with shared group type characteristics. For example, mixed serous and endometrioid tumours. There is uncertainty on how these variants of cancers develop and how they may progress, therefore creating issues over their classification. Along with the mixed variants, there is confusion over carcinosarcomas (malignant mixed mullerian tumours, or MMMTs) as they are currently excluded from the uterine sarcoma and classified as metaplastic carcinomas (D'Angelo & Prat 2010). MMMTs account for 1-2% of all uterine corpus malignancies (Lax 2007). Endometrial carcinomas and MMMTs have distinct molecular genetic pathways and

exhibit different biological features, however, MMMTs epithelial component can resemble high grade endometrioid, serous or clear cell carcinoma. In light of this, many studies classify carcinosarcomas as NEECs (Lax 2007; Okuda et al 2010, Kernochan & Garcia 2009).

Whether it is by understanding the underlying genetic changes in tumour development and progression or examining the clinical and pathological features of the several types of endometrial cancer; further studies on endometrial cancer classification are required.

Endometrial cancer is largely diagnosed histologically from endometrial tissue obtained from a pipelle or other forms of endometrial biopsy. A systematic quantitative review and a meta-analysis evaluating the accuracy of pipelle biopsies for the diagnosis of endometrial cancers and atypical hyperplasia showed it to be 81-99% sensitive and 98% specific (Dijkhuizen et al 2000; Clark et al 2002). Transvaginal ultrasound can also be used to identify symptomatic women who may have a diagnosis of endometrial cancer, identifying a thickened and irregular endometrial lining which may be associated with the growth of endometrial cancer cells.

All diagnosed cancers are staged surgically or from clinical findings and scans. International Federation of Gynecology and Obstetrics (FIGO) recently developed a new staging scheme for endometrial cancers. It incorporates depth of myometrial invasion and extra-uterine metastatic disease (Saso et al 2011). See Table 2.

Table 2: FIGO staging of endometrial cancer

FIGO Stage	Description
Stage IA	Tumour confined to the uterus, no or < ½ myometrial invasion
Stage IB	Tumour confined to the uterus, > ½ myometrial invasion
Stage II	Cervical stromal invasion, but not beyond the uterus
Stage IIIA	Tumour invading serosa or adnexa
Stage IIIB	Vaginal and/or parametrial metastasis
Stage IIIC1	Metastasis to pelvic lymph nodes
Stage IIIC2	Metastasis to pelvic and para-aortic lymph nodes
Stage IVA	Tumour invasion of the bladder and/or bowel mucosa
Stage IVB	Distant metastasis including abdominal and/or inguinal lymph nodes

Histological grading only applies to endometrioid endometrial adenocarcinomas, other non-endometrioid carcinomas including serous and clear-cell are automatically classed as grade 3. Cancer is classed as low or high grade by the assessment of the percentage of solid growth, pattern if invasion and presence of tumour necrosis (Amant et al 2005). According to FIGO histological differentiation of endometrioid cancer is as follows (Table 3):

Table 3: FIGO grading of endometrial cancer

FIGO Grade	Description
Grade 1	< 5% of solid/non-glandular areas
Grade 2	6-50% of solid/non-glandular areas
Grade 3	> 50% of solid/non-glandular areas

The differentiation of endometrial cancers is one of the most important prognostic factors. Grade 1, 2, and 3 tumors make up approximately 45%, 35%, and 20%, respectively, of adenocarcinomas of the endometrium. The 5-year survival rate of clinical stage I cancers is 94%, 88%, and 79% for grade 1, 2, and 3 tumors, respectively. (Chiang JW, E Medicine).

Less histological differentiation is associated with a higher incidence of deep (ie, greater than one half) myometrial invasion and lymph node metastases. Subsequently, the depth of myometrial invasion and presence of tumor in the lymph nodes is directly related to recurrence rates and 5-year survival rates. (Chiang JW, E Medicine)

Primary treatment for advanced metastatic disease is surgery whereas radio- or chemotherapy is offered subsequently. Improving the current understanding of the metastatic process may not only provide new target for improved therapeutic success but will also provide means of acquiring additional diagnostic information on the metastatic potential of the cancer at the first diagnosis of endometrial cancer to plan more focused treatment.

1.3 METASTASIS

Metastasis is a cascade of linked steps involving cancerous cells, originating from a primary tumour with the ability to invade surrounding tissue and disseminate to distance ectopic sites (Mendoza & Khanna 2009; Liotta & Stetler-Stevenson 1991). This is quite a common process that occurs in many cancers in their advanced stage. The distant spread and invasion (metastasis) of cancerous endometrial cells beyond the uterine cavity contributes to the advanced disease and is the reason for death in endometrial cancer. This is despite the significant improvements in diagnosis, surgical techniques and general patient care.

Various chemokines, proteins, enzymes, immune cells etc are implicated in cancer metastasis, yet the exact mechanism of this spread at a cellular level is poorly understood. Understanding the pathogenesis of metastasis on the systemic, cellular and molecular levels are important goals of cancer research.

There have been many theories and hypotheses made explaining this complex and intricate process. The "seed and soil" hypothesis is a well-known theory first described by Stephen Paget in 1889, suggesting that metastasis relies on interaction between selected cancer cells (the seeds) and specific organ microenvironments (the soil).

Definition of the seed and soil hypothesis consists of three principles:

1. Primary neoplasms (and metastases) consist of both tumour cells and host cells. Host cells include epithelial cells, fibroblasts, endothelial cells and

infiltrating leukocytes. Moreover, neoplasms are biologically heterogeneous and contain genotypically and phenotypically diverse subpopulations of tumour cells, each of which have the potential to complete some steps in the metastatic process, but not all.

2. Process of metastasis is selective for cells that succeed in:
    a. Invasion
    b. Embolization
    c. Survival in the circulation
    d. Arrest in a distant capillary bed
    e. Extravasation into the organ parenchyma
    f. Multiplication within the organ parenchyma

3. Metastases can only develop in specific organs. The microenvironments of different organs (the soil) are biologically unique. Outcome of metastasis depends on multiple interactions (cross-talk) of metastasizing cells with homeostatic mechanisms, which the tumour cells can usurp. Therapy of metastases, therefore, should be targeted not only against the homeostatic factors that promote tumour-cell growth, survival, angiogenesis, invasion and metastasis.

James Ewing challenged this theory in the 1920's proposing that the circulatory system was primarily responsible for the organ-specific metastatic dissemination. Both have been shown to be equally important and interlinked over time as research in this field expands.

Weiss and Sugarbaker concluded regional metastasis involved anatomical or mechanical considerations, such as efferent venous circulation or lymphatic drainage to regional nodes. On the other hand distant metastasis were site specific. The distribution of metastatic lesions is largely dependant on mechanical factors, e.g. arrest of emboli in capillaries of secondary organs, therefore suggesting both mechanical and the 'soil' factors are required for metastasis to occur.

The main sites of metastasis are liver, lung, peritoneum and bone, the occurrence of which depends on the primary location of the cancer. Spread of endometrial cancer cells originating from the lining of the uterus initially migrate and penetrate into the myometrium. At this stage it becomes more likely to spread to local areas via the lymphatic system, beginning with malignant cells infiltrating local pelvic, paraaortic and inguinal lymph glands (Morrison et al 2009). The cancer can grow into the fallopian tubes, cervix and vagina, or further beyond into the bladder or rectum. In advance, late stage malignancy cancer cells can penetrate through the outer lining of the uterus (serosa) and spread into the abdomen and affecting the peritoneum.

1.4 METASTASIS INDUCING PROTEINS (MIPs)

Endometrial cancer is an oestrogen dependent disease. In breast carcinoma, which is also an oestrogen dependent disease, expression of a panel of proteins predict the survival and that they were shown to be involved in metastasis (Wang et al 2006; Tuck & Chambers 2001; Rodrigues et al 2007, De Silva Rudland et al 2006; Rudland et al 2000; Jenkinson et al 2004; Fritzsche et al 2006; Innes et al 2006; Liu et al 2005). These proteins are called metastasis inducing proteins (MIPs) and include S100P, S100A4, Osteopontin (OPN) and Anterior gradient homolog 2 (AGR2). The work carried out included in this thesis proposed to investigate if there are similarities in the expression of these proteins in endometrial cancer, and if there is a correlation between FIGO cancer grading and the expression of the MIPs suggesting that they may play a similar role as in breast cancer.

1.4.a INVOLVEMENT OF MIPs IN ENDOMETRIAL CANCER

To the best of my knowledge, there are only a few studies exploring the metastatic process in endometrial cancer. Five publications have suggested that OPN and S100A4 are expressed by primary and cell line endometrial cancer cells (Du et al 2009; Cho et al 2009; Hashiguchi et al 2006; Xie et al 2009; Xie et al 2007). However, the expression of the other two MIPs, namely S100P and AGR2 has yet to be explored in human endometrial cancer.

A study by Du et al (2009) showed an over-expression of OPN in a human endometrial cancer cell line Ishikawa, (ISK) and in tumour-associated human

endometrial endothelial cells (HEECs) isolated from tissue samples of a patient with endometrial cancer. They conducted a follow up study using the short interference RNA (siRNA) method to silence the OPN gene in HEECs and ISK cells. Knocking down OPN gene reduced the cell migration and invasiveness of these cancer cells in vitro. In a mouse model of tumourigenesis they demonstrated that and decreased invasiveness in both HEECs and ISK compared to normal controls. Furthermore, ISK cells transfected with OPN siRNA formed a smaller tumour in a mouse model of tumourigenesis compared to the scrambled siRNA controls. This data suggested an important role of OPN in endometrial cancer-growth and associated angiogenesis. (Du et al 2009).

Cho HB et al (2009) using real-time polymerase chain reaction and immunohistochemistry evaluated the role of OPN as a potential biomarker in endometrial cancer. Expression of OPN was first analysed in endometrial cancer cell lines and endometrial cancer tissue, both of which showed an over expression of OPN compared to normal endometrium. Plasma OPN levels were measured, showing a significantly higher preoperative level of OPN in endometrial cancer patients than in healthy controls. Patients were also followed up and monitored for survival and recurrence (mean time 22.1 months), which displayed that the overall survival and disease-free survival rates for OPN positive patients were significantly lower than the survival rates of OPN negative patients. These results have demonstrated the potential value of plasma OPN as a useful diagnostic marker for endometrial cancer (Cho et al 2009).

Approximately 10% of patients with ovarian cancer develop endometrial cancer synchronously, and 5% vice versa (Chiang et al 2008). Herrinton et al (2001) suggested that these tumours may be mechanistically linked to reproductive hormones. However, it is unclear whether this is in relation to primary tumours or to metastasis from the ovary to the endometrial tumour or vice versa (Soliman et al 2004, Zaino et al 2001). Most ovarian tumours are derived from the surface epithelium of the ovary and are adenocarcinomas of different histological subtypes, manifesting in various morphological forms as (cyst) adenocarcinomas with serous, mucinous, clear-cell, or endometrioid differentiations (Hemminki et al 2003, Young & Scully 2002). As discussed in Chapter 1.2, primary endometrial neoplasms also include the same subtypes as Type 1 or Type 2 endometrial carcinomas. Van Niekerk et al (2009) carried out a study investigating the association between primary endometrioid carcinoma of the ovary and synchronous malignancy of the endometrium. Their results indicated that in 2.9% of patients diagnosed with epithelial ovarian malignancy a second new primary malignant tumour is occurring in the endometrium, especially women diagnosed with an endometrioid histological subtype.

In a study comparing OPN expression at DNA, RNA and protein levels of endometrioid endometrial cancer and ovarian endometrioid cancer tissue, OPN expression was seen in 50% (15/30) of the endometrioid endometrial cancers. There was no significant difference observed in the percentage of positive cytoplasmic OPN staining between the ovarian and endometrial cancer cells. Ovarian endometrioid cancer is known to resemble endometrioid endometrial cancer morphologically, however, they behave different biologically (Zwart et al 1998). This study aimed to

investigate whether the different behaviours of the cancers could be explained by OPN expression, but this hypothesis could not be accepted (Hashiguchi et al 2006).

Comparison between the behaviour of ovarian and endometrial cancers, in light of their morphological similarities is an interesting area of research. Hashiguchi et al (2006) broached the idea of OPN involvement in the association between the two cancers. This should hopefully stimulate further molecular studies into the possibly carcinogenic pathways and widen our knowledge on the role of OPN and the other MIPs.

Both studies examining the role of S100A4 in endometrial cancer showed an over-expression of S100A4 in high-grade and advanced stage endometrial carcinomas. Xie et al (2009) down-regulated S100A4 expression in endometrial cancer cell line, which resulted in undetectable S100A4 protein level and significantly decreased migration and invasion. Both studies showed the important relevance and role of S100A4 in endometrial cancer, especially in advanced stage cancer where the chance of developing metastasis is high. This may suggest that S100A4 plays a vital role in the metastasis of endometrial cancer.

A recent study has shown that these proteins are cyclically expressed in the normal human endometrium and are aberrantly expressed in women with endometriosis, which is a benign metastatic disease (Hapangama et al 2011 (In press)). This gives us a good basis to further examine whether endometrial cancer is associated with abnormal expression of MIPs and weather MIPs are associated with advanced disease, and decrease survival.

1.4.a.i OSTEOPONTIN

Osteopontin has been found to be produced by epithelial cells of the gastrointestinal, urinary and reproductive tracts, the gall bladder, pancreas, lung bronchi, lactating breast, salivary glands and sweat ducts (Rodrigues et al 2007).

Senger was first to describe Osteopontin as a glycol-phosphoprotein that is expressed by numerous human malignant epithelial cells in 1979 (Senger et al 1979). OPN gene has been mapped to chromosome 4q13 in humans, and along with its associated isoforms the molecular weight is measured to be between 41-75 kDA. It is also know to be expressed at high levels by T-lymphocytes, epidermal cells, bone cells, macrophages, and endothelial cells (Wai & Kuo 2004; Chakraborty et al 2006).

Various investigators have examined the functional role of osteopontin further, to reveal that it functions by mediating cell-matrix interactions and cellular signaling through binding with intregrin and CD44 receptors. OPN facilitates cell adhesion, chemotaxis, stress-dependent angiogenesis, prevention of apoptosis, and anchorage-independent growth of tumor cells.

OPN is a ligand for several integrins, especially integrin $\alpha_v\beta_3$, and since integrins are involved in cell-cell adhesion and anchorage, it is not surprising that integrin $\alpha_v\beta_3$ and OPN have been detected in breast cancer bone-metastases. This OPN:$\alpha_v\beta_3$ interaction directly mediates migration and invasion of tumor cells, and induces neovascularization by up-regulating endothelial cell migration, survival, and lumen formation during angiogenesis. OPN also facilitates cell-matrix and cell-cell interactions by the interaction via CD44 glycoproteins which are universally expressed cell-surface adhesion molecules.

Recent studies have provided substantial evidence to link OPN with the regulation of metastasis, by establishing a correlation between high levels of OPN expression and malignant invasion in various cancers, including breast, stomach, lung, prostate, liver and colon (Shevde et al 2010; El-Tanani et al 2006; Furger et al 2001).

1.4.a.ii S100P

S100P was found to be up regulated during the implantation window in the human endometrium (Tong et al 2010). However, the role that S100P plays in the implantation window is still unclear. Most S100 proteins are thought to mediate regulatory functions by binding to and modulating the biological activity of intracellular compounds (Tong et al 2010).

S100P is a 95-amino acid member of the large S100 family, small calcium binding proteins with intracellular or extracelluar functions. Becker et al (1992) founded the protein, which was purified and characterized from the placenta, designating the 'P". S100 was denoted to identify a group of proteins soluble in a 100% saturated ammonium sulfate solution (Moore 1965). While all S100 genes are localized on human chromosome 1, the gene for S100P is exclusively located on human chromosome 4q16. (Schafer et al 1995).

There is increasing evidence suggesting that S100P has a significant role in cancer. This gene has been found to be expressed in several cancers, including pancreas, colon, prostate, and lung. It has also been shown to be associated with poor clinical outcome. (Logsdon et al 2003, Guerreiro Da Silva et al 2000, Wang et al 2006, Fuentes et al 2007, Mousses et al 2002, Diederichs et al 2004).

S100P has been shown to mediate tumor growth, drug resistance, and metastasis. Therefore, S100P is a useful marker for differentiating cancer cells from normal cells and can aid in the diagnosis of diagnosis by cytological examination.

Studies in breast cancer, an oestrogen dependent cancer like endometrial cancer, have shown there to be a link between expression of S100P and progression of breast cancer (Guerreiro Da Silva et al 2000). There has also been evidence to support the role of S100P as an inducer of breast cancer metastasis.

There is no research investigating the expression of S100P in endometrial cancer, but it has been hypothesized that endometrial cancer will have a similar expression of S100P to breast cancer as they are both oestrogen dependent cancers.

The primary mechanism of action of S100P, at least in the case of pancreatic and colon cancer, is through the activation of the cell surface receptor RAGE (Receptor for advanced glycation end products). (Arumugam et al 2010). RAGE can be activated by a number of ligands, including advanced glycation end products, specific S100 molecules (S100B, S100A12, and S100P), amyloid, and amphoterin (Logsdon et al 2007; Sparvero et al 2009). RAGE participates in a number of important pathological processed besides cancer, including Alzheimer's diseases, diabetes, and inflammation (Logsdon et al 2007; Sparvero et al 2009).

Activation of RAGE by S100P stimulates cellular signaling pathways, including the mitogen-activated protein (MAP) kinase and nuclear factor-B (NFkB) pathways. NFkB signaling may be of particular importance, because elevated NFkB activity is associated with increased resistance to therapies in different cancers, Therefore, interventions that block the ability of S100P to activate RAGE may provide therapeutic benefit.

1.4.a.iii ANTERIOR GRADIENT 2

Anterior gradient homolog 2 (AGR2) was first described in *Xenopuslaevis* embryos, as it played an important role in inducing the formation of the forebrain and the mucus secreting cement gland. It was then identified in humans, during the investigation focused on differentially expressed genes in oestrogen receptor-positive breast cancers (Wang et al 2008).

The first study reporting the expression of AGR2 in human endometrium arises from our laboratory (Hapangama et al 2011). A cyclical variation in the expression of AGR2 in the normal fertile control samples and increased expression of AGR2 in endometriosis samples was observed. Endometriosis is a benign metastatic disease, which has similar properties and functions as cancerous metastasis. This provides us with a foundation to hypothesis that AGR2 would be over-expressed in endometrial cancer.

It is an oestrogen and androgen responsive protein, up regulated in a number of cancers, including breast, lung, ovarian, gastric, pancreatic, oesophageal, and prostate. Its expression in all premalignant and malignant oesophageal adenocarcinoma suggests that it serves an important role in disease pathogenesis (Wang et al 2008).

Extensive research has been carried out investigating the role of AGR2 in breast cancer tissue and cell lines (Fritzsche et al 2006; Innes et al 2006; Liu et al 2005;Wang et al 2008). Endometrial cancer is also an oestrogen dependent disease, like breast cancer, therefore AGR2 may express abundantly in endometrial cancer.

1.4.a.iv S100A4

There are now over twenty S100 family members that are localised to specific tissue in humans, a multi-gene family of calcium binding proteins of the EF-hand type. S100A4 gene in humans is located in the region of chromosome 1q21, which is frequently rearranged in a number of malignancies, including endometrial cancer. Several investigators cloned the S100A4 gene, identifying the protein as a highly expressed transcript in growth-stimulated cultured cells, metastatic tumor cell lines and during morphogenic conversion from an epithelial to mesenchymal phenotype. In addition, during oncogenic transformation S100A4 expression levels are up regulated (Garrett et al 2006; Helfman et al 2005; Xie et al 2009).

The association between S100A4 and metastatic capacity was first introduced by Ebralidze et al in 1989. Since then S100A4 has been categorized as a metastasis associated protein and a regulator of tumor progression, with significant over-expression seen in breast, pancreatic, prostate, gallbladder, esophageal, gastric, lung, and thyroid carcinomas in comparison with its normal tissue (Garrett et al 2006; Rudland et al 2000).

Studies to determine the function of S100A4 have shown that it may play a role in the different aspects of tumor progression, including motility, invasion, and apoptosis. It has been proposed that S100A4 affects motility through its interaction with a non-muscle myosin. It is also involved in a variety of cellular processes, such as immune response, differentiation, cell growth etc (Ismail et al 2010; Sherbe 2009; Tarabykina et al 2007; Zhang et al 2005).

S100A4 has also been coined to be a prognostic marker in a number of cancers, as expression of S100A4 is highly correlated with patient death. The universality of

S100A4 expression in a variety of cancers illustrates the potential use of S100A4 as a marker for tumor metastasis and disease progression (Garrett et al 2006), which hopefully can be applied for endometrial cancer.

1.5 HYPOTHESES

Since the four MIPs have shown to be involved in the metastatic process of breast cancer, studies are under way to investigate if they would be suitable therapeutic targets to prevent distant spread of breast cancer. Therefore, it is important that we assess the involvement of the same proteins in endometrial carcinogenesis and metastasis as the advances in treatment of breast cancer can then hopefully be exploited in the treatment of endometrial cancer as well.

With the literature to date on endometrial cancer and MIPs we propose the following:

1. The literature in breast cancer studies suggests that MIPs are expressed in breast cancer cells. Breast cancer and endometrial cancer, in particular Type 1 endometrioid adenocarcinomas are both oestrogen dependent diseases. Therefore we hypothesise that the four metastasis-inducing proteins AGR-2, S100A4, S100P, Osteopontin are expressed in endometrial cancer cells.

2. Since the oestrogen dependence of various endometrial cancer types are different, we hypothesise that there is differential MIPs expression in these tumour types.

3. Higher the grade and wide spread cancer, increases the risk of metastatic disease, therefore we hypothesise that high-grade endometrial cancers e.g. Type 2 cancers, have an increased expression of the MIPs in comparison to low-grade tumours

2 METHODS

Samples used in this study were obtained from human subjects. A vast number of ethical issues arise from collecting human samples for the purpose of research. These were all taken in consideration and appropriate ethical approval was sought from the adult local research ethics committee in Liverpool before commencing the study. We collected samples on two different ethically approved projects. Control samples, normal fertile and postmenopausal endometrium were collected under "Endometrial stem cell study" ethics (09/H1005/55). Endometrial cancer samples, and few postmenopausal control samples were collected under "MIPs in endometrial cancer study" ethics (11/H1005/4). [Refer to Appendix 7.1 & 7.2 for consent forms and patients information leaflets for each ethics].

2.1 PATIENT RECRUITMENT

Patients were recruited from the Liverpool Women's Hospital. Consent from patients was obtained by a trained member of the research team or by doctors from the Liverpool Women's Hospital.

We employed a strict inclusion and exclusion criteria in order to recruit necessary patients for the study. (See Tables 4 & 5).

Table 4: Principle inclusion criteria

Study Group	Women with a histological diagnosis of endometrial cancer, undergoing surgical treatment.
Normal Control Group	A) Normal ovulating, fertile women in the proliferative phase of their menstrual cycle, undergoing surgical procedure for benign conditions (e.g. laparoscopic sterilization). B) Post-menopausal women undergoing hysterectomy with NO history of abnormal bleeding.

Table 5: Principal exclusion criteria

Study Group	History of other gynecological cancers (cervical, ovarian etc).
Normal Control Group	• Women in either the menstruation or secretory phase of their menstrual cycle. • History of abnormal vaginal bleeding. • Women who have take hormonal contraceptives over preceding three months, including with an intra-uterine contraceptive device. • Women currently breast feeding or pregnant. • History of infertility or recurrent pregnancy loss. • History of endometriosis.

Patients who fit the above criteria and scheduled for surgery were approached on the day of the operation and informed of the study. They were given a patient information leaflet, explaining the reasons for the study, their involvement and contact details.

Patients who chose to consent to having an endometrial biopsy and blood sample taken were asked to sign a consent form. Two copies of the consent form were taken, one kept in the patient's notes and the other, secure in the research department with the sample and blood processing paper work.

Additional clinical information was gained from the patient and their notes, including (Table 6):

Table 6: Patient demographic data

General	• Age • Weight, height & BMI • Smoker/Non-smoker • Last menstrual period/Beginning of menopause • Cycle length • Gravida/Parity • Relevant past medical history, e.g. Hypertension, Diabetes mellitus • Relevant gynecological history, e.g. Gynae malignancies or operations
Endometrial Cancer Patients	• Histological diagnosis • Staging of the cancer • Presenting complaint • Treatment plan (Chemotherapy/Radiotherapy)

2.2 ENDOMETRIAL SAMPLE COLLECTION AND PROCESSING

No extra surgical procedures were carried out over and above which was clinically indicated in any women in the study. Either a pipelle sample or full thickness sample was obtained from patients during theatre.

Endometrium was obtained using a pipelle from women who were not having a hysterectomy. Trained members of the research team or the surgeons obtained pipelle

samples from the uterus in situ prior to the plan procedure. Pipelle sampling is a routine procedure undertaken in day clinics and usually not under anesthetic. A pipelle is a flexible plastic tube that allows a sample of endometrium to be obtained. In theatre, the patient was prepped and sterilised for their planned surgery as normal. The pipelle was removed from its sterile packaging and carefully inserted through the cervical canal into the uterus, following which the pipelle's plunger was withdrawn. Whilst rotating and gently inserting the pipelle in and out of the uterine cavity an adequate amount of tissue from the endometrium should be suctioned out.

Full thickness endometrial samples were obtained from patients having a hysterectomy. Shortly after the uterus was removed, orientation of the uterus was determined and placed on its anterior side. Using a carbon steel surgical blade, a median incision on the posterior side was carefully made from the fundus to the cervical canal. Deeper incisions were made to expose the uterine cavity adequately. Once the endometrium was identified, a curved lateral incision was made along side the endometrium. At either ends of this incision, two further incisions were made at 90 degrees through the myometrium to dissect out a full thickness sample.

The endometrial biopsies collected were divided into four. The first part was frozen immediately for protein/RNA extraction, the second was placed in neutral buffered formalin (NBF) for paraffin sections, the third in collection media for culturing, and the final part in RNA later for RNA extraction (Refer to SOP in appendix 7.3a).

2.2.a FROZEN SAMPLES

Extracted endometrium was immersed into phosphate buffered saline (PBS) to remove excess blood from the sample, to ensure only tissue is frozen. The sample was

transferred to the laboratory and placed on dry ice during the preparation time under the fume hood. Using forceps the entire sample was transferred onto a disposable plastic container. All viable tissue was then carefully placed into a cyrotube, labeled with the sample ID and date and stored in a -70°C freezer.

2.2.b PARAFFIN SAMPLES

In order to obtain paraffin sections of samples, one part of the endometrial sample was directly placed in 10% NBF solution and incubated for a minimum 24 hours at room temperature. NBF is a special fixative solution that preserves the tissue and cells, as they are normally found in situ and causes the cross linking of proteins.

Samples are further embedded in wax to allow thin sections of the sample to be cut. This was achieved by processing the samples through a well-cycled program incubating them in increasing concentrations of alcohol from 60% to 100%, xylene and finally hot paraffin wax. These steps ensured the removal of water within the tissue, and subsequently replaced with paraffin wax. Once processed the sample was embedded in liquid paraffin using the embedding machine, allowed to set and stored in the fridge at -4°C. Embedding samples in paraffin maintains the natural shape and architecture of the sample during long term storage and sectioning. (See SOP in appendix 7.3c & 7.3d)

2.2.c CULTURE SAMPLES

A section of all the endometrial samples collected was immersed in collection media, so an attempt could be made to culture and grow the cells. The collection media

provides the cells with the perfect environment and essential nutrients to adequately grow and develop.

2.2.d RNA LATER SAMPLES

A small section of the endometrium was placed in a cyrotube containing RNA later solution for a minimum 24 hours at -4°C. The sample was then transferred into a fresh cyrotube and stored in the freezer at -70°C.

2.3 BLOOD SAMPLE COLLECTION AND PROCESSING

Blood samples were also taken from all patients. This was taken in the anaesthetic room prior to the surgery, from the same cannula placed by the anaesthetists to gain venous access. A total of 10ml of blood was taken and dispersed into blood serum and ethylenediaminetetraacetic acid (EDTA) collection tubes.

The whole blood sample is directly aliquotted into to small cyrotubes. Plasma serum blood bottle was centrifuged and top plasma serum layer was then transferred into cyrotubes. Both blood samples are frozen and stored in a -70^{o}C freezer. (See SOP in appendix 7.3b).

2.4 SECTIONING AND SLIDE PREPARATION

After samples were processed and embedded as explained in chapter section 2.2.b, thin sections of the samples are required for the immunohistochemistry technique. Using the Microm rotary microtome (Microm Ltds, Thame, UK) the paraffin blocks were sectioned with a 3um thickness. The sections were carefully floated onto a pre-heated water bath and picked up onto aminopropyl triethoxy saline (APES) coated microscope slides. Slides were dried overnight at room temperature before immunohistochemistry steps were followed. (Refer to SOP in appendix 7.3.e)

2.5 LABORATORY TECHNIQUES

To assess the expression of MIPs in endometrial cancer tissue techniques such as immunohistochemistry and RT-PCR were employed.

2.5.a IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) is a technique that uses antibodies to microscopically identify, localize and stain proteins (antigens) in tissue samples. A set protocol was discussed and created by the research team to ensure the best possible outcome. It was then employed to assess the expression of the four proteins (S100P, OPN, S100A4 and AGR-2) using the Vector ImmPRESS IHC reagent kit. (See SOP in appendix 7.3.f & 7.3.g)

2.5.a.i METHOD

Slides used were clearly labeled with information of the antibody tested, its conditions and the date. They were then baked for an hour at 60°C or overnight at 36°C to soften wax and ensure the tissue remains on the slide. Paraffin wax must be completely removed from the slide to ensure aqueous reagents adhere and penetrate the tissue; this was achieved by immersing the slides in xylene for 2 x 10 minutes. Tissues sections were then re-hydrated through graded ethanol to water as followed: 2 x 5 minutes in 100% ethanol, 1 minute in 70% and further minute in 60% ethanol followed by immersion in distilled water.

A pH 6 0.01M citrate based solution was used in the antigen retrieval procedure and was achieved by pressure-cooking the sections for one minute, otherwise known as the heat induced epitope retrieval (HIER) method. Antigen retrieval is designed to unmask antigens in the paraffin embedded tissue sections by breaking protein cross-links, which were created during paraffin fixation by NBF. This therefore enhances the intensity of antibody detection. The use of heat in the HIER method interrupts the cross-links while buffer solution aid in maintaining the break allowing the antibody to attach onto its paired antigen.

Following antigen retrieval, slides were washed in tris buffered saline (TBS) for 5 minutes, and incubated in 0.3% hydrogen peroxide/TBS for 10 minutes blocking any endogenous peroxidase activity in the specimen. TBS is a buffer used to maintain the samples pH levels between 7-9.2. Endogenous peroxidase and alkaline phosphatase are the endogenous enzymes found in a variety of tissues and most commonly known to generate false positive signals. The use of hydrogen peroxide on the samples increases the level of substrate hence eliminating endogenous enzyme activity.

Slides were washed again in TBS for 5 minutes. A DAKO hydrophobic marker pen was used to mark the area on the tissue section to be stained, followed by another 5 minute TBS wash.

Sections were incubated in a moisture chamber, to prevent the sections form drying out with the primary antibodies presented in table 7:

Table 7: IHC Antibody conditions

Antibody	**Concentration**	**Duration**	**Temperature**
Polyclonal rabbit anti-human AGR-2 (Liu et al., 2005)	1:100	Overnight	4˚C
Monoclonal rabbit anti-human S100A4 (DAKO Ltd, UK)	1:2000	Overnight	4˚C
Monoclonal rabbit anti-human Osteopontin (Developmental Studies Hybridoma Bank, Iowa City, IA, USA)	1:500	2 hours	Room temp.
Monoclonal mouse anti-human S100P (BD Transduction Laboratories, Cowley Oxford, UK)	1:100	2 hours	Room temp.

The relevant antibody dilutions were made using 0.5% bovine serum albumen (BSA). At higher temperatures the antigen-antibody pairing occurs quicker, therefore to prevent false over expression of proteins overnight incubations were stored at a lower temperature of 4°C.

Prior to the application of osteopontin antibody, horse serum was applied to all sections selected for osteopontin staining for 20 minutes at room temperature. This is used to reduce background or non-specific staining.

Sections were then thoroughly washed in TBS twice for 5 minutes. Slides were placed back in the humidified chamber and incubated with secondary antibody for 30 minutes at room temperature. Anti-rabbit biotinylated horseradish peroxidase (HRP) polymer was used for anti-S100A4, AGR-2 and Osteopontin and anti-mouse HRP polymer for S100P antibody.

After the final two washes in TBS, sections were stained with a substrate/chromagen solution using ImmPRESS polymerized reporter enzyme staining system. The diaminobenzidine (DAB) solution was applied onto the anti-S100A4 and AGR-2 for 5 minutes; and for 10 minutes for the anti-S100P and Osteopontin sections. All sections were then washed in distilled water to stop the DAB reaction and counterstained with filtered Gill 2 haematoxylin for a minute. Sections were dehydrated through graded ethanols and xylene, times as follows: 1 minute in 60% ethanol, 1 minute in 70%, 2 x 3 minutes in 100%, 5 minutes in xylene and further 10 minutes in fresh pot of xylene. Coverslips were mounted over each section using DAKO faramount aqueous mounting medium, and left to dry before analyzing under light microscope.

Negative controls anti-rabbit and anti-mouse IgGs were included in each staining run. Breast carcinoma sections were used as external positive controls for the four MIPs.

2.5.a.ii IHC ANALYSIS

Immunohistochemistry was used to assess the expression of the four MIPs in endometrial cancer in comparison to the two control groups, normal fertile and post-menopausal endometrium.

Quick Score technique was used to semi-quantitatively analyse and compare the IHC staining for the 4 MIPs, assessing 10 high power fields per sample also taking in

consideration the size and integrity of each sample. The amount of staining and the intensity was assessed in each area and multiplied together, obtaining a Quick Score value as previously described by Detre S et al (1995) and Schiessl et al (2008). An average of the Quick Scores for each high power field per sample were used in the analysis. When calculating the expression seen in glandular epithelium and luminal epithelium, following scoring system was used (Table 8):

Table 8: Quick Score for glandular and luminal epithelium

Intensity		**Proportion of Stain (%)**	
0	Absent	1	< 25%
1	Weak	2	25 – 50 %
2	Moderate	3	50 – 75 %
3	Strong	4	> 75 %

For the assessment of stromal staining, the numbers of stromal cells positively stained per high power field were counted and so the proportion of stain was altered accordingly (Table 9):

Table 9: Quick Score for stromal cells

Intensity		**Cell Count** **(No. of positive cells/field)**	
0	Absent	1	< 10
1	Weak	2	11 – 20
2	Moderate	3	21 – 30
3	Strong	4	> 30

2.5.b REVERSE TRANSCRIPTION – POLYMERASE CHAIN REACTION

2.5.b.i INTRODUCTION

Polymerase chain reaction (PCR) is a unique molecular biological technique used to amplify and reproduce defined sections of precise genetic material. It involves manipulating the action of a naturally occurring enzyme, Taq DNA polymerase, a member of a family of proteins that ensure accurate replication of all living matter. DNA polymerases are biological macromolecules that catalyse the formation and repair of DNA. In the 1980s, Kary Mullis at Cetus Corporation discovered a way to start and stop the action of polymerase at specific points along a single strand of DNA using two primers complementary to sequences at the left and right ends of the region of DNA to be amplified. Further experimentation led Mullis to the discovery that by harnessing this component of molecular reproduction technology, the target DNA could be exponentially amplified; otherwise known as a chain reaction, allowing unlimited quantities of specific DNA to be replicated.

There are three fundamental steps involved in PCR:

1. **DENATURING (MELTING):**

   Double stranded DNA samples are heated at 94-96°C to separate the two strands of DNA by breaking the hydrogen bonds between the two strands. For this study samples were denatured at 95°C for 15 minutes prior to the first cycle to ensure that both the template DNA and primers are separated. Samples were then further heated at 94°C for 15 seconds per cycle.

2. **ANNEALING:**

   Once the strands have been separated, the temperature is lowered to 50-65°C allowing the left and right primers to anneal (attach) to their complementary single strand sequences. The primers are designed to bracket the DNA region that is required to be amplified. The temperature of this stage depends on the working temperature of the primer, which is usually 5°C under the melting temperature. Primer binding will not occur if the temperature is set too high, and a lower temperature would results in non-specific binding. In this study samples were heated at 60°C for 45 seconds allowing the annealing of the primers to the single strands of DNA template to occur optimally.

3. **EXTENSION:**

   This final step is carried out at 72°C for 1 minute, which is the ideal working temperature for the Taq DNA polymerase enzyme. Polymerase attaches to each priming site and incorporates complementary nucleotides into the single DNA strand (i.e. when the polymerase reads a "G" on the template strand it adds a "C", likewise "A-T", "G – C" and "T – A"). Therefore creating a copy of the DNA template of the region specified by the annealed primers.

These three steps are repeated 20-40 times, the exact number of cycles depends on the conditions set to obtain desirable results. With one cycle each single strand of a double stranded DNA template has been amplified into two separate double-stranded DNA, which, after strand separation, are then available as a template for further amplification in the next cycle. This process leads to exponential amplification of the original DNA template (See Figure 1).

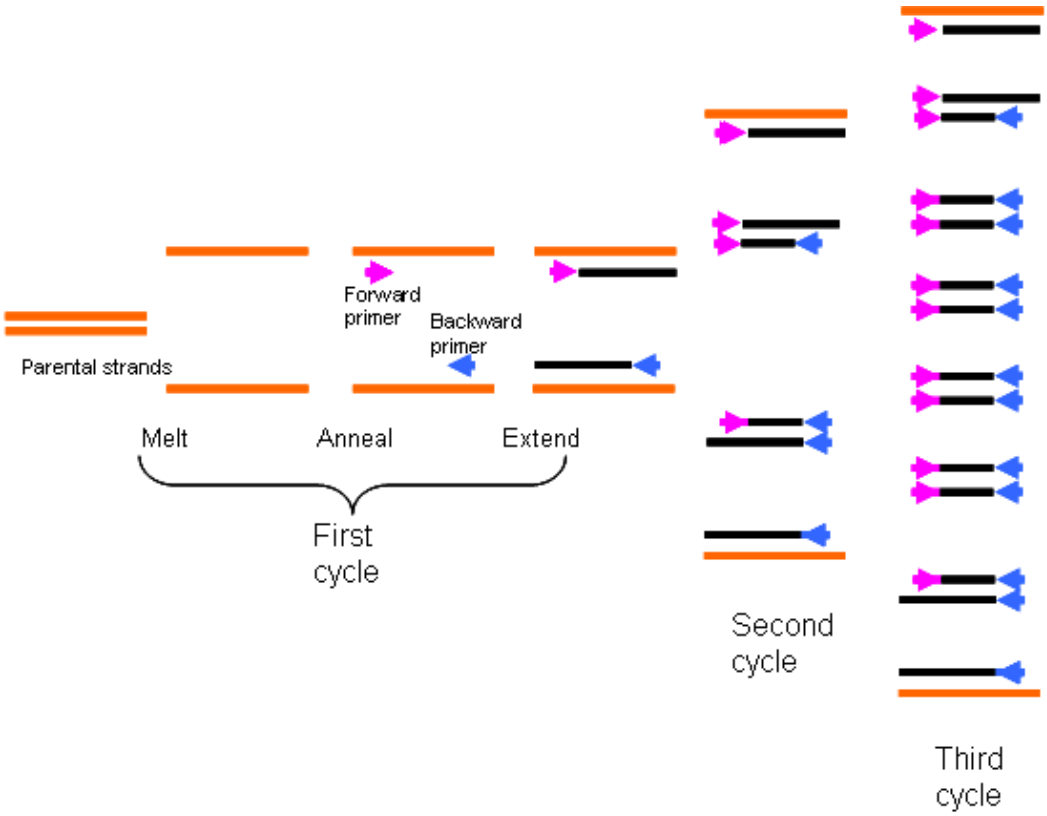


Figure 1: PCR Steps (Adapted from http://www.korinfo.hu/drupal/en/node/8912)

Reverse transcription – polymerase chain reaction (RT-PCR) is a variant of the PCR technique in which RNA strands are used as the starting material. An additional step is performed to convert the RNA to complementary DNA (cDNA) using the enzyme reverse transcriptase, prior to the PCR steps as described above. This technique was used in this study to investigate the expression of the MIP mRNAs.

2.5.b.ii METHOD

2.5.b.ii.1 RNA EXTRACTION

The first step involved extraction of RNA from the endometrial tissue samples. For extraction of RNA from formalin-fixed, paraffin embedded (FFPE) tissue samples a QuickExtract FFPE RNA Extraction Kit (Epicentre Biotechnologies, Cambridge,

United Kingdom) was used. This kit only requires heat to ensure that RNA is amplified accordingly. Tissue sections were heated along with QuickExtract FFPE RNA Extraction Solution in a thermocycler for 30 minutes at 56°C, and then 98°C for 2 minutes. This melted the paraffin, disrupted the cells, decreased the formalin-induced cross-linking in the sample and degraded compounds that may inhibit amplification. Further DNAse treatment was undertaken on the samples to improve the quality of amplification and prevent DNA being amplified directly by the Taq DNA polymerase. This was achieved by adding RNAse-free DNAse 1 to the extracted RNA samples, and incubation at 37°C for 10 minutes. Finally, Stop Solution was added to the sample tubes and heated to 65°C for 10 minutes to terminate the DNAse activity.

For extraction of total RNA from frozen tissue, cultured cells and RNA-later samples, a TRIzol Plus RNA Purification Kit (Invitrogen Ltd., Paisley, United Kingdom) was used. TRIzol Reagent is a monophasic solution of phenol, guanidine isothiocyanate, and other proprietary components, which facilitate the isolation of RNA. It maintains the integrity of the RNA, while disrupting cells and dissolving cell components, as well as providing an immediate and highly effective inhibition of RNAse activity during sample homogenization or lysis.

Tissues and RNA-later samples were homogenized in 1mL TRIzol Reagent produce a cell lysate and cultured cells were directly lysed in the culture dish by adding 1mL TRIzol Reagent. The resulting cell lysate was passed several times through a pipette tip to shear the DNA. 0.2mL of chloroform was added to the sample and the mixture was centrifuged at 12,000 x g for 15 minutes at 4°C to separate the solution into an upper aqueous phase containing the RNA and a lower phenol-containing organic phase. The upper aqueous phase (~ 600uL) is transferred to a new RNAse-free tube,

followed by the addition of an equal volume of 70% (v/v) ethanol to obtain a final ethanol concentration of 35% and the solution centrifuged.

The sample was then loaded onto a PureLink RNA Mini Kit Spin Cartridge containing a silica-based membrane to which the RNA binds during purification. Loading involved transferring about 700uL of the prepared sample to a Spin Cartridge using a pipette and centrifuging at 12,000 x g for 15 seconds at room temperature. The flow-through was discarded and the Spin Cartridge was reinserted into the same collection tube. The above steps were repeated until the entire sample has been processed. For washing and elution, 700uL Wash Buffer I was added to the Spin Cartridge and centrifuged at 12,000 x g for 15 seconds at room temperature. The flow-through was discarded with the Collection Tube and Spin Cartridge was inserted into a new Collection Tube. 500uL of Wash Buffer II containing ethanol was added to the tube and centrifuged at 12,000 x g for 15 seconds at room temperature twice, discarding the flow-through after centrifuging. The Spin Cartridge and Collection Tube was centrifuged again at 12,000 x g for 1 minute at room temperature to dry the membrane with attached RNA. The Collection Tube was discarded and the Spin Cartridge was inserted into a Recovery Tube. 30uL of Rnase-free water was added to the centre of the Spin Cartridge and left for 1 minute. This step was repeated twice, adding a total of 90uL of Rnase-free water. Finally the Spin Cartridge was centrifuged with the Recovery Tube for 2 minutes at 12,000 x g at room temperature. The Spin Cartridge was discarded, leaving the Recovery Tube, which contains the purified total RNA and was stored at -80°C until required.

2.5.b.ii.2 CALCULATING RNA CONCENTRATION

The RNA concentration for each sample was measured in a cuvette using the Soft Max Pro 5.2 programme, on the settings:

Wavelength: 2

Limit 1: 260 nm

Limit 2: 280 nm

First a blank was tested (i.e. no RNA) in order to obtain a reference for the other samples. 60uL of distilled water, or enough to adequately cover the cuvette window, was added to the cuvette and placed in the machine. The Soft Max Pro 5.2 programme is opened and "Reference" followed by "Read" is clicked giving the result:

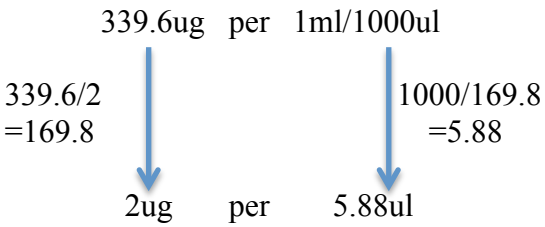
A1
0.000 OD {OD = Optical density}
0.000 OD

RNA samples were then measured, using 2uL of total RNA and 58uL of distilled water, ensuring that the cuvette was thoroughly cleaned with distilled water and dried between testing different samples to prevent contamination. The 260/280 optical density ratio was calculated (Limit 1/Limit 2). A ration of >1.8 is considered satisfactory, but ratios below 1.8 (lowest 1.55) were allowed for this study, due to the limited number of samples being tested. The RNA concentration [ug/mL] for each sample was also calculated (OD_{260} x 40ug/ml x dilution value), where the dilution value is 60uL/2uL. Around 2-4ug of total RNA was required to be reverse transcribed.

The volume of RNA concentration is calculated like so, using the example below:

Sample A: RNA concentration calculated as 339.6ug/ml

Need 2ug/ml RNA of Sample A for 1st C-DNA synthesis.



Therefore, 5.9ul of Sample RNA is required for its 1st C-DNA synthesis

2.5.b.ii.3 1ST C-DNA SYNTHESIS

Total RNA was reverse transcribed using SuperScript reverse transcriptase III (Invitrogen Ltd., Paisley, United Kingdom in a final volume of 20ul containing PCR-Water, OligodT (0.5ug/ul), RNA Inhibitor, Total RNA (2-4ug), 5x1st cDNA Buffer, 0.1M DTT and 10nM dNTP mix.

All reagents were completely dissolved, vortexed and centrifuged before pipetting and reactions were set up on ice using 0.5uL autoclaved PCR tubes. Autoclaved PCR tubes were clearly labeled with sample ID and date. The following reagents were added to each PCR tube in this order (Table 10):

Table 10: Solutions and amounts used for 1st step of 1st cDNA synthesis

Solutions	**Sample 1 (ul)**	**Sample 2 (ul)**	**Sample 3 (ul)**
PCR-H_20	x	x	x
OligodT (0.5ug/ul)	1	1	1
RNAse inhibitor	0.5	0.5	0.5
Total RNA (~2ug)	Y	Y	Y
Total Volume	9	9	9

OligodT and RNAse Inhibitor volumes are fixed and total RNA volume (Y) is obtained from the calculation above. To get the total volume of 9ul, PCR-H_20 volume (X) was altered accordingly.

All tubes were vortexed and centrifuged for 1 minute. They were then placed in the PCR machine, and heated at 70°C for 10 minutes. Samples were then cooled on ice and centrifuged.

A master mix of the following solutions was prepared: [For Y reactions, prepare (Y+1) master mix]. (See Table 11)

Table 11:Master mix solutions & calculation for 2nd step of 1st cDNA synthesis

Solutions	**1x Reaction (ul)**	**>3 Reactions [Y+1] (ul)**
PCR-H_20	3	3x [y+1]
5x1st cDNA Buffer	4	4x [y+1]
0.1M DTT	2	2x [y+1]
10mM dNTP mix	1	1x [y+1]
Total Volume	10	10 x [y+1]

Master mix was well mixed by vortexing and centrifuged, before aliquotting 10ul of solution to each sample tube. Finally 1ul of mmLV-RT superscript reverse transcriptase was added to each of the tube to get a total volume of 20ul in each tube. All tubes are vortexed and centrifuged and placed in the PCR machine. Samples were incubated at 50°C for 1 hour and again for 15 minutes at 70°C. 1st strand cDNA reaction mixtures were then stored at -20°C until required for the PCR reactions.

2.5.b.ii.4 PCR

The first-strand cDNA reaction mixture (1 ul) was amplified by PCR with Taq DNA polymerase (Qiagen) as previously described (Liu *et al.,* 2005).

> Solutions used: PCR-Water, AGR-2, S100A4, OPN & S100P 5' and 3'-Primers, Qiagen Taq DNA polymerase mix (5xQ Solution, 10xPCR buffer, dNTPmix, polymerase, [with addition DMSO for OPN and S100P]).

New 0.5ul autoclaved PCR tubes were labeled with the sample ID and date. A total volume of 10ul, 1:10 dilution of 1st the cDNA-containing solution was made. Solutions were heated at 98°C for 10 minutes in the thermal cycler, followed by placing on ice for two minutes and centrifuged.

A master mix of the relevant primers were made and added to the mix (table 12):

Table 12: Primer master mix calculation used for PCR reaction

Solution	**1x Reaction (ul)**	**>3x Reaction (ul) [y+1]**
PCR-H_20	13	13x [y+1]
5'-Primer	1	1x [y+1]
3'-Primer	1	1x [y+1]
Total Volume	15	15 [y+1]

The master mix primer solution was mixed, centrifuged briefly and 15uL was aliquoted to each sample tube. Finally 25ul of the Qiagen PCR master mix (2x) was added to each tube. All samples were vortexed, centrifuged and placed in the thermal cycler to be heated using the programme:

95^0C – 15mins

94^0C – 30secs
60^0C – 45secs } 26 cycles
72^0C – 1min

70^0C – 10mins
4^0C – Hold

GenBank accession numbers, primer position and sequence, and product sizes are presented in table 13 below. Human glyceraldehyde-3-phosphate dehydrogenase (GPDH) was used to provide a normalization control.

Table 13: List of PCR primers used for RT-PCR

Gene Name	**Accession number**	**Sequences [5' -> 3']**		**size**	**Tm [^{0}C]**	**PCR product size [bp]**
Human AGR2	NM_006408	Fwd 5' 217	5' GCT CCT TGT GGC CCT CTC CTA CAC 3'	24	60	354
		Rev 3' 570	5' ATC CTG GGG ACA TAC TGG CCA TCA G 3'	25	60	
Human GPDH	NM_002046	Fwd 5' 628	5' ACC ACA GTC CAT GCC ATC AC 3'	20	66	452
		Rev 3' 1079	5' TCC ACC ACC CTG TTG CTG TA 3'	20	67	
Human OPN	NM_001040058	Fwd 5' 562	5' GTC ACT GAT TTT CCC ACG GAC CTG CC 3'	26	76	282
		Rev 3' 843	5' TTC ATA ACT GTC CTT CCC ACG GCT GT 3'	26	73	
Human S100A4	NM_002961	Fwd 5' 70	5' ATG GCG TGC CCT CTG GAG AAG G 3'	22	74	306
		Rev 3' 375	5' TCA TTT CTT CCT GGG CTG CTT A 3'	22	67	
Human S100P	NM_005980	Fwd 5' 211	5' GGA GCT ACC AGG CTT CCT GCA GAG TGG 3'	27	76	180
		Rev 3' 390	5' CCA GGG CAT CAT TTG AGT CCT GCC 3'	24	75	

2.5.b.ii.5 AGAROSE GEL ELECTROPHORESIS

PCR products were visualized with SYBR Safe (Invitrogen Ltd., Paisley, United Kingdom) or Ethidium Bromide. PCR product identity was confirmed by a correct sized band by agarose gel electrophoresis and DNA sequence analysis of the PCR product.

0.5g of agarose powder was added to 50ml of 1xTAE buffer (Tris base, EDTA and acetic acid (pH8.0)) and heated in a microwave. The solution was left to cool to about 60°C. Once cooled, stock of either 5ul ethidium bromide (10mg/ml) or 2ul SYBR Safe was added to the agarose mixture. Ethidium bromide and SYBR safe intercalated into the double stranded cDNA and allows for visualization under ultraviolet light. The mixture was then gently mixed, poured into the gel tray without delay and allowed to set for 30 minutes. A gel comb was placed across the top of the gel to create wells in order to place the cDNA samples. While the gel was setting, about 500mL of electrophoresis buffer (1xTAE buffer) was poured into a electrophoresis tank, enough to cover the agarose gel by 1-2mm. 1ul of loading buffer and 4ul of the PCR product was added to individual sample tubes using fresh pipette tips for each sample to prevent cross-contamination. All tubes were vortexed and centrifuged, prior to transfer into the wells. For each set of samples for the four MIP's, a negative and positive control was included.

Once the gel was set, the comb was removed and the gel tray was carefully placed in an electrophoresis tank, which was filled with electrophoresis buffer. 5ul of the sample was transferred into each well. 1uL of the DNA ladder (molecular weight marker) was then placed at the end in one of the wells. It was important to avoid

making any mistakes in recording the order of the samples and to use new clean pipette tips for each sample, control or marker to prevent contamination.

Once all the samples were transferred, the lid was placed on the electrophoretic tank and its electrodes were connected to electrophoresis current supply unit and run at about 90-100 volts for 30-40 min. The gel was then removed from the electrophoresis tank and placed on a UV transilluminator and a photograph of the gel was taken. This photograph helped to verify the presence of the PCR products and hence was used for the quantification process.

2.5.b.iii RT-PCR ANALYSIS

PCR bands produced from RT-PCR technique were quantified using ImageJ software (Version 1.44o, USA). The pixel intensity of each column was calculated, creating a histogram for each the values, with pixel location on the x-axis and intensity value on the y-axis (See example in figure 2). The area under the curve represents the amount of signal in the band. For all the MIPs, the area of each band (i.e. sample) was calculated three times, from which the average was then calculated and used for further analysis.

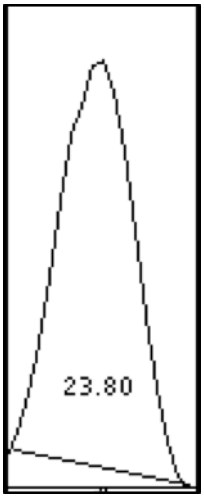


Figure 2: Example of a histogram plot generated from quantifying a mRNA sample using Image J

Normalisation of target gene expression levels must be performed to compensate intra- and inter-kinetic RT-PCR variations (sample-to-sample and run-to-run variations). All samples were normalised against their corresponding GAPDH PCR band, using the calculation: **AX x (G1/GY)** Where X is variable. See table 14 for examples:

AX: Sample to be normalised, e.g. AGR2 Endo Ca A
G1: GAPDH Endo Ca Sample A (used for all other AGR2 samples in the sample set)
GX: GAPDH sample being normalized.

Table 14: Normalisation calculation for quantifying PCR bands

Samples	Calculation
Sample A	Sample A x (GAPDH A/GAPDH A)
Sample B	Sample B x (GAPDH A/GAPDH B
Sample C	Sample C x (GAPDH A/GAPDH C)

Once normalized values were calculated, data was statistically analysed.

2.6 STATISTICAL ANALYSIS

All data used were analysed using Graph Pad Prism 5 for Mac, GraphPad software (version 5.0d, USA). Two normal control groups (Normal Fertile Control and Post Menopausal Control) were used to directly compare Endometrial Cancer immunohistochemistry results. Non-parametric Mann Whitney test, which does not assume Gaussian distribution, was used to compare Endometrial Cancer samples with the two control groups independently. We further analysed the Normal Fertile Control group compared with the Post Menopausal Control group to ascertain any difference between those two independent groups. Pearson's correlation test, which assumes Gaussian distribution was also, used to ascertain an association between Endometrial Cancer and FIGO grade.

Normal Fertile Control samples were compared with Endometrial Cancer samples for PCR, along with a positive internal control using endometriosis samples. Non-parametric Kruskal-Wallis test, which does not assume Gaussian distribution, was used to compare PCR results of Endometrial Cancer, Normal Fertile Control and Endometriosis (positive internal) Control groups.

Data with a p value of < 0.05 was deemed as statistically significant and p value of <0.1 as a trend between <0.1 and <0.05.

3 RT-PCR OPTIMIZATION

Reverse transcriptase polymerase chain reaction is the most sensitive technique for mRNA detection and quantification. In this study it was used as an alternative confirmatory test to our IHC study of endometrial MIPs.

RNA was extracted from a total of 22 endometrial samples for the analysis of Osteopontin, S100P, S100A4 and AGR2 expression by RT-PCR. Two control groups were included for the comparison with endometrial cancer samples, normal fertile and as a positive internal control, endometriosis samples. We were not able to extract RNA from postmenopausal endometrial pipelle biopsies due to insufficient quality or quantity.

From the RNA samples extracted, successful results for all the MIPs were obtained and analysed from eight sample; two endometrial cancers, three normal fertile and three endometriosis samples. This was again due to either the insufficient quality or quantity of RNA in the samples collected.

3.1 RNA QUALITY TEST

To test which RNA extraction method would the most successful, an endometrial cancer sample (A) with stored frozen and paraffin embedded samples was chosen. This sample was also cultured for several months, successfully creating a primary cell

line. The RNA extracted from the cultured cells, if successfully amplified, would be used as an internal control. RNA was therefore extracted from the cultured cells, frozen and sections of the paraffin embedded sample, using the two different methods explained in the previous chapter.

RNA concentrations from all three variants of the endometrial cancer sample (A) were calculated. The optical density ratio was calculated:

Cultured Cells = 1.931

Frozen = 2.01

Paraffin = 0.893

An optical density ratio of >1.8 is considered satisfactory; therefore we eliminated the RNA extracted from the paraffin sample for this PCR run.

AGR-2 primer was used to test the two samples, including GAPDH to provide a normalization control. 1st c-DNA reaction mixture was made for each sample and amplified by PCR on 29 cycle programme. Agarose gel electrophoresis results are show below in Figure 3:

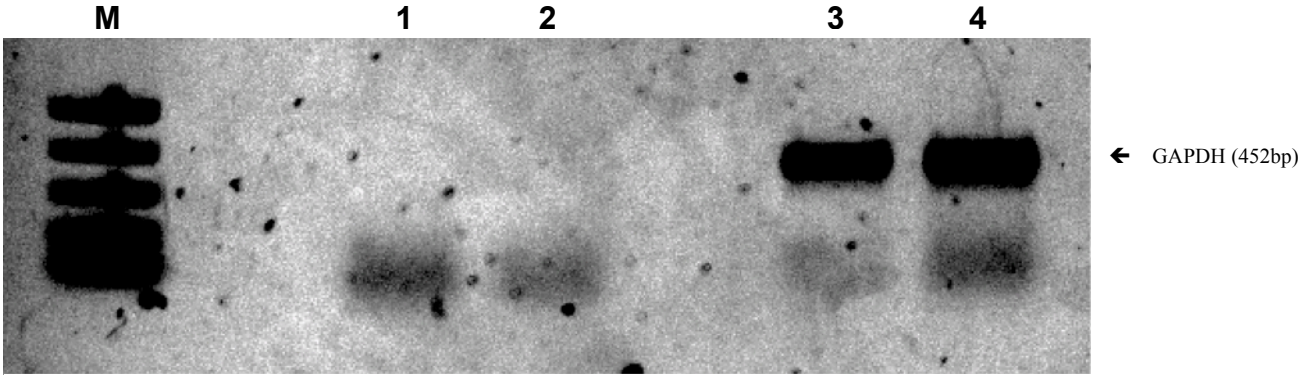


Figure 3: GAPDH and AGR2 testing culture cells and frozen section of an endometrial cancer sample A. Lane M: DNA ladder, Lane 1: AGR2 Culture Cells, Lane 2: Frozen, Lane 3: GAPDH Cultured Cells, 4: GAPDH Frozen

Results show that both frozen and culture cell samples were negative for AGR2. However a positive control was not included in this run, so the result seen could not be verified.

A PCR was run testing the culture cells, frozen and paraffin endometrial cancer samples, including a positive control for AGR2 (MCF7 breast cell line). As the optical density ratio was only 0.893 for the paraffin, various conditions were examined. Two different 1st c-DNA reaction mixtures were made for AGR2 and GAPDH, one with 2ug of total RNA and other with 4ug. Both c-DNA mixtures were then amplified at 30 cycles and 40 cycles. Hence testing 2µg at 30 cycles, 2µg at 40 cycles and 4µg at 30 cycles, 4µg at 40 cycles. See Figure 4 for the gel picture:

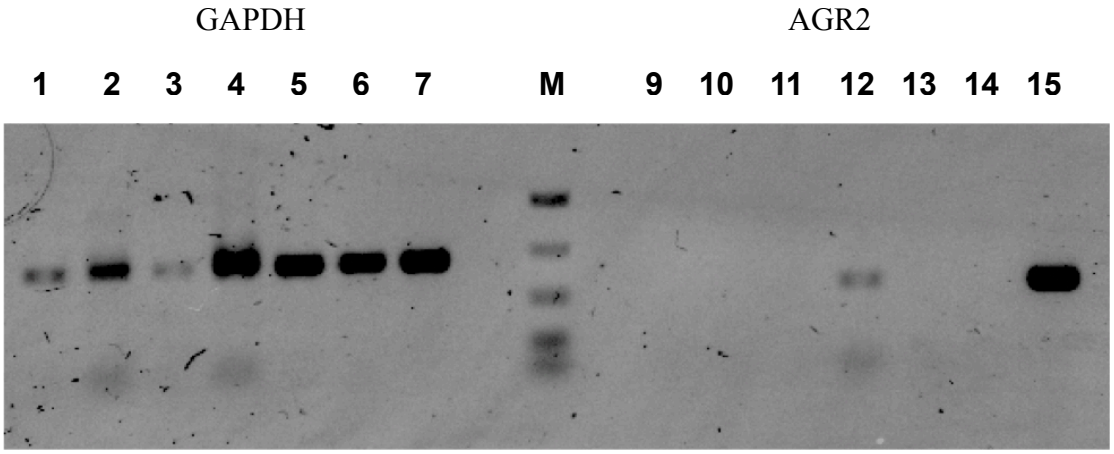


Figure 4: GAPDH PCR for testing different RNA extraction methods on endometrial cancer sample (A). 1-7: GAPDH, 9-15: AGR2. 1 & 9: Paraffin 2µg 30 cycles, 2 & 10: Paraffin 2µg 40 cycles, 3 & 11: Paraffin 4µg 30 cycles, 4 & 12: Paraffin 4µg 40 cycles, 5 & 13: Frozen tissue, 6 & 14: Culture cells, 7 & 15: MCF7 positive control.

The frozen tissue and culture cells were still negative for AGR2, in comparison to the MCF7 positive control. 4µg of total paraffin RNA at 40 cycles amplified enough RNA for a band to be visible. GAPDH band for 4µg paraffin at 40 cycles has also been amplified the most compared to the other conditions set for the paraffin sections.

Results therefore suggest that RNA extraction from paraffin sections is a suitable method, which can be used for the PCR amplification of other samples.

Following this RNA was extracted from sections of a further 10 paraffin embedded endometrial tissue sample (B-K), three normal fertile, four endometriosis and three endometrial cancer samples. GAPDH and AGR2 primers were tested on these sample, see gel pictures Fig 5 and 6 below:

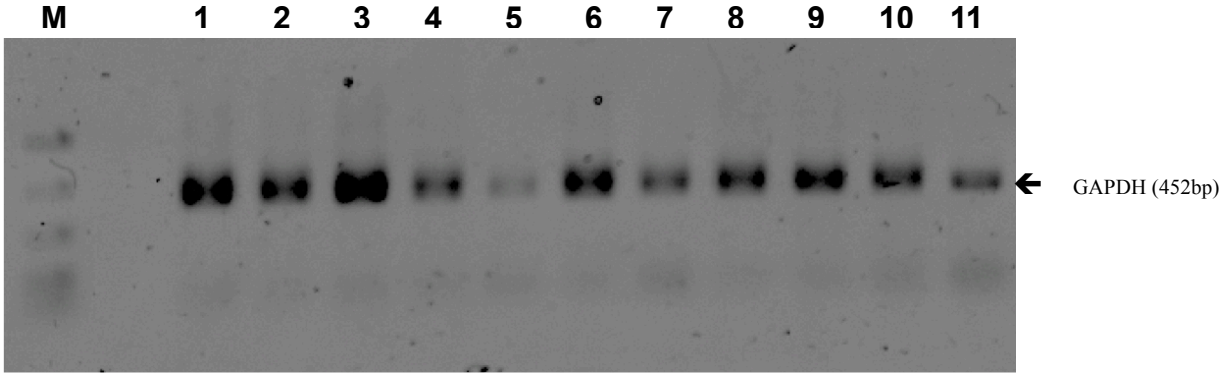


Figure 5: GAPDH PCR for samples B-K. Lane M: DNA Ladder, 1-3: Normal fertile, 4-7: Endometriosis, 8-10: Endo Cancer, 11: Positive control

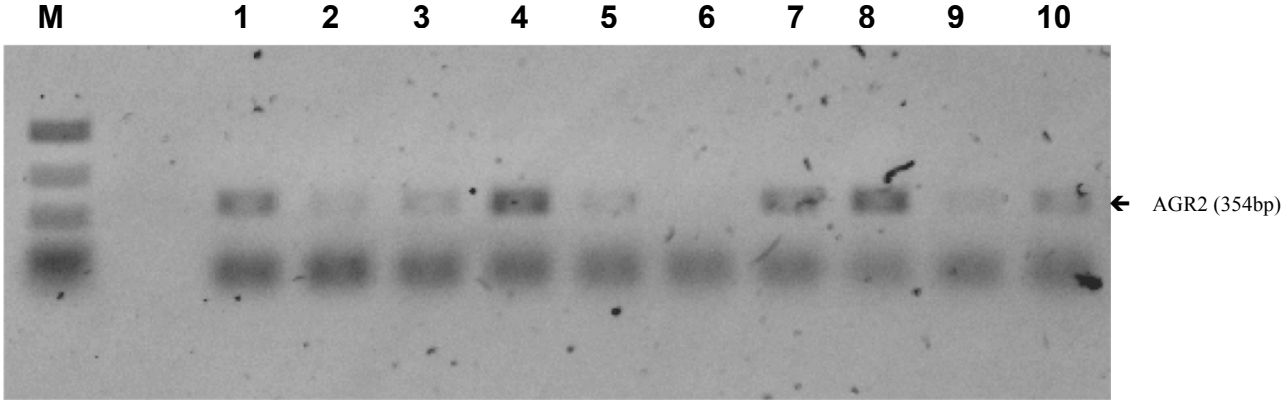


Figure 6: AGR2 for samples B-K. Lane M: DNA ladder, Lanes 1-3: Normal fertile control, Lanes 4-7: Endometriosis and Lanes 8-10: Endometrial cancer.

There is a high level of primer dimer amplified below the RNA sample, which can be due to various reasons including the use of non-purified RNA samples, reduced cycle length etc.

A clear difference was seen between the three different groups for the expression of AGR2, so the 11 samples (A-K) were analysed for the expression of S100A4, S100P and OPN.

Results showed varied positive and negative expression for S100P in the RNA A-K samples, however the RNA of the samples were not amplified for the expression of S100A4 or OPN. See Fig 7-9:

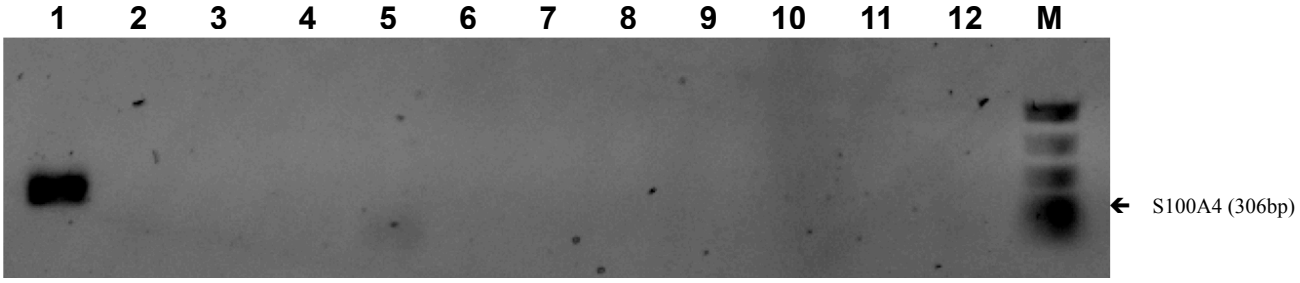


Figure 7: S100A4 expression for sample A-K, 1: Positive control, 2: Negative control, 3-5: Normal fertile control, Lanes 6-9: Endometriosis and Lanes 10-12: Endometrial cancer, Lane M: DNA ladder.

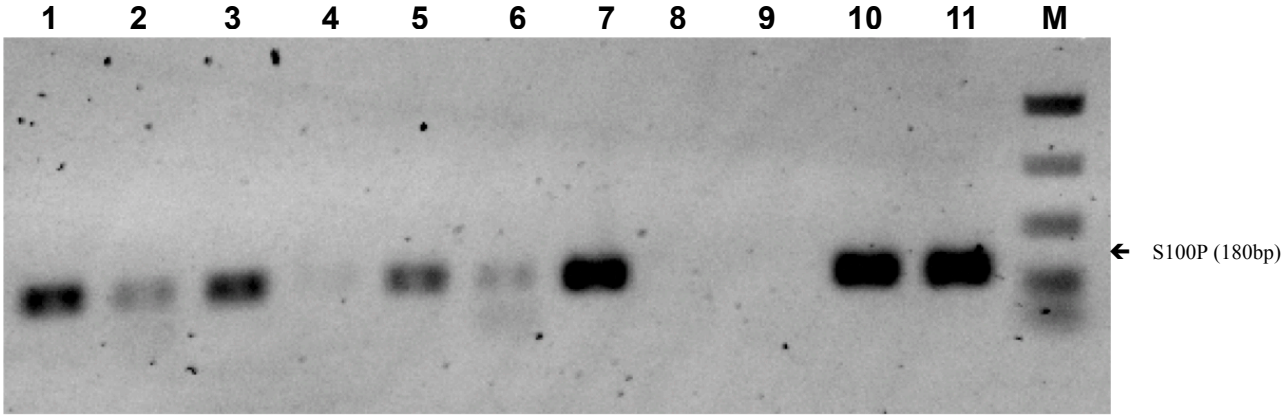


Figure 8: S100P expression for samples A-K. Lane 1-3: Normal fertile, 4-7: Endometriosis, 8-11: Endometrial cancer, M: DNA ladder.

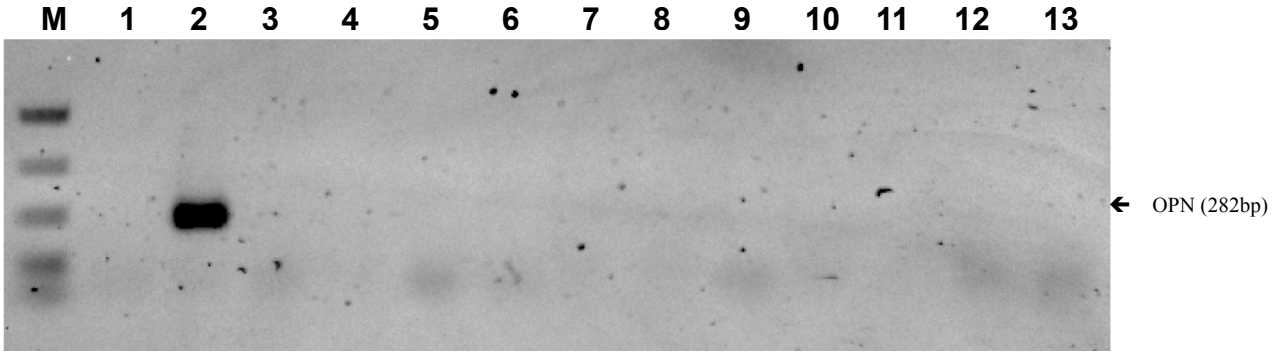


Figure 9: S100A4 expression for sample A-K. Lane M: DNA ladder, 1: Negative control, 2: Positive control, 3-5: Normal fertile control, Lanes 6-9: Endometriosis and Lanes 10-13: Endometrial cancer.

The paraffin sections used for the samples may have not extracted enough RNA for S100A4 or OPN to amplify, therefore different samples were chosen to analyse. RNA was extracted from the frozen or RNA later sections for the new set of samples. This method was chosen, as it is known to produce a more purified concentration of total RNA.

3.2 PCR CONDITION TESTING

RNA was extracted from a new set of 11 endometrial samples, three normal fertile, five endometriosis and three endometrial cancers. RNA concentration was calculated for each sample, but three samples (one endometriosis and two endometrial cancers) were far too dilute to be used and removed from the new set.

The RNAs (L-V) were amplified with a 40cycle programme examining the expression of AGR2 and GAPDH as a normalization control. Gel electrophoresis observed an over amplification of the RNA for both AGR2 and GAPDH. See Fig 10 and 11. This initiated a re-evaluation of the conditions used for the PCR amplification of these samples, as the RNA is more purified compared to the paraffin sections.

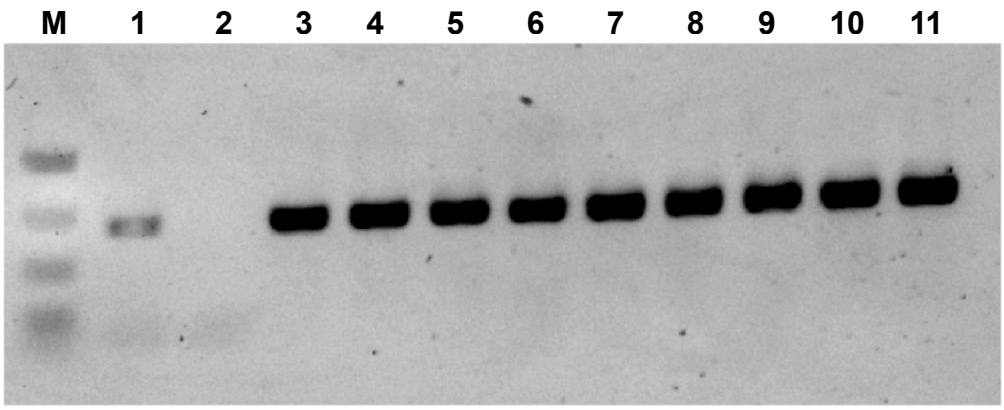


Figure 10: GAPDH expression for samples L-V. Lane M: DNA Marker, 1: Positive control, 2: Negative control, 3-5: Normal fertile, 6-9: Endometriosis, 10-11: Endometrial cancer.

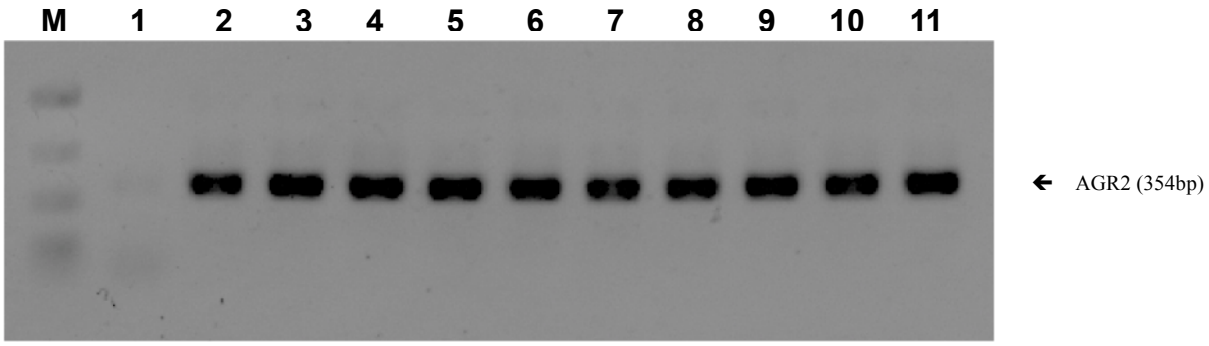


Figure 11: AGR2 expression for samples L-V. Lane M: DNA Marker, 1: Positive control, 2: Negative control, 3-5: Normal fertile, 6-9: Endometriosis, 10-11: Endometrial cancer.

The cycle length was reduced to 30 cycles to assess whether it altered the amplification. One normal fertile (L), one endometriosis (O) and two endometrial cancer samples (U&V) were tested for S100A4 and OPN at 30 and 40 cycles. Results showed that the cycle length need to be further reduced. See Fig 12:

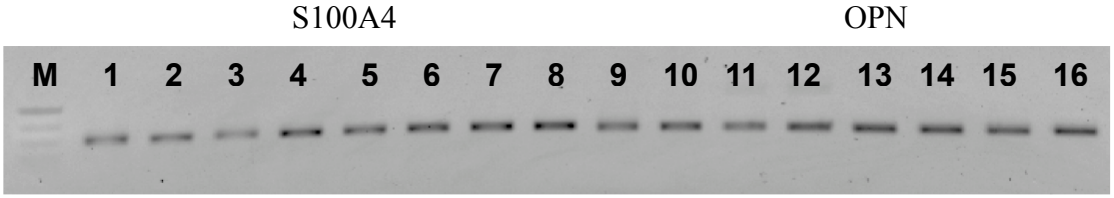


Figure 12: Testing S100A4 and OPN on New RNA samples (L, O, U & V) at 30cycles and 40cycles. Lane M: DNA marker, 1-8: S100A4, 9-16: OPN. 1: Normal (L) 30C, 2: Normal (L) 40C, 3:Endometriosis (O) 30C, 4: Endometriosis (O) 40C, 5: Endometrial cancer (U) 30C, 6: Endometrial cancer (U) 40C. 7: Endometrial cancer (V) 30C, 8: Endometrial cancer (V) 40C. Repeat for 9-16 OPN.

A further PCR run was done assessing the sample at cycles 20, 22, 24 and 28 using the endometriosis sample (O) and endometrial cancer sample (U). This revealed that the optimum cycle length for S100A4 and OPN would be 26 cycles. See Fig 13:

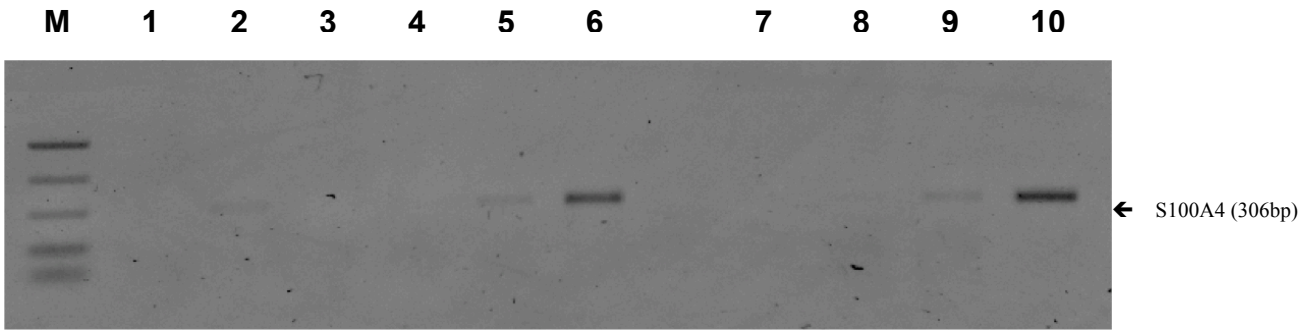


Figure 13: Testing S100A4 on new RNA sample (O & U). Lane M: DNA Ladder, 1: Negative control, 2: Positive control, 3-6: Endometriosis sample (0) 3: 20 cycle, 4: 22 cycle, 5: 24 cycle, 6: 28 cycle. Repeat for 7-10 for Endometrial cancer sample U.

Once the optimal conditions were established for the four MIPs for the new set of RNA. RT-PCR was run to assess the expression of the four MIPs in normal and endometrial cancer. The next chapter (Results) will discuss and analyse the results further.

4 RESULTS

This study was completed in approximately twelve months period from September 2010 to August 2011 and used a total of 55 endometrial samples, 30 from women with endometrial cancer, 15 from normal fertile women with regular menstrual cycles and 10 from post-menopausal women. Functionalis layer of the endometrium was collected with pipelle endometrial sampler hence only the functional layer of the endometrium is analysed for the purpose of this study.

4.1 PATIENT DEMOGRAPHICS

Over 80 endometrial samples were collected from normal fertile women over the last 12 months. All samples were histologically dated according to modified Noyes criteria (Murray et al 2004), also taking in to consideration of the last menstrual period according to the woman's recollection and her declared regularity of the cycle. We included two normal control groups to directly compare with the endometrial cancer samples. Since most women develop endometrial cancer in the postmenopausal period, postmenopausal endometrial samples were collected and used a control group. In the normal fertile women, we chose to study only endometrial samples that were assigned to the proliferative phase of the cycle as another control group. Previous literature reports both these endometrial sample groups being used to compare with endometrial cancer samples as control groups, hence our choice to include both groups.

4.1.a DEMOGRAPHIC DETAILS

Fertile women included in the study had no history of endometriosis or pathological abnormality of the endometrium. They were undergoing laparoscopic sterilization or unrelated operation, and were not on any hormonal treatments. Likewise for the post-menopausal women recruited, participants had no history of abnormal bleeding or endometrial pathology. They were not on any hormonal replacement therapy and underwent hysterectomies for prolapse.

As expected the pre-menopausal women were younger than the other two groups (KW test, $p < 0.0001$, see Table 15). BMI was higher in patients with endometrial cancer compared the two control groups, but not statistically different. (KW test, $p = 0.0971$). In three patients I was unable to find the demographic information to include in this analysis.

Table 15: Mean age and BMI of individuals in each study group

Study Groups	**Mean Age (±SD)**	**Mean BMI (±SD)**
Normal Fertile	40.20 ± 4.97	28.44 ± 5.99
Post Menopause	66.70 ± 7.48	28.24 ± 3.93
Endometrial Cancer	66.82 ± 9.46	32.23 ± 7.50

4.1.b ENDOMETRIAL CANCER TYPES

As expected 81.5% of women in our study had endometrioid adenocarcinoma, 11% with endometrial carcinosarcoma and 7.4% with mixed variants (mixed serous and endometrioid adenocarcinoma). I was unable to retrieve the detailed pathology report describing the cancer type for 3 patients. (See Table 16)

Table 16: Grouping cancer type

Cancer Type	Sample Numbers	Percentage (%)
Endometrioid endometrial adenocarcinoma	22	81.5
Carcinosarcoma	3	11
Other	2	7.4
Detailed pathology report unavailable	3	11

Histological grading only applies to endometrioid endometrial adenocarcinomas, other non-endometrioid carcinomas including serous and clear-cell are automatically classed as grade 3. International Federation of Gynecology and Obstetrics (FIGO) grading scheme was used to grade the endometrioid endometrial adenocarcinomas as described in chapter one. 50% of the endometrioid endometrial cancer sample set was FIGO grade 1 endometrioid endometrial adenocarcinoma (See table 17)

Table 17: Grouping FIGO grade for endometrioid endometrial adenocarcinoma

FIGO Grade	Sample Number	Percentage (%)
1	11	50
2	8	36.4
3	3	13.6

All diagnosed cancers are staged using the FIGO staging scheme as described in chapter one. FIGO stage data was retrieved from the pathology reports for 25 of the endometrial cancers in my study group. 52% of women with endometrial cancer were diagnosed at an early stage (IA) and subsequently treated with a hysterectomy (See table 18).

Table 18: FIGO Stage of all cancers

FIGO Stage	Sample Number	Percentage (%)
IA	13	52
IB	6	24
II	4	16
IIIA	0	0
Stage IIIB	2	8
Stage IIIC1	0	0
Stage IIIC2	0	0
Stage IVA	0	0
Stage IVB	0	0

4.2 IMMUNOHISTOCHEMISTRY

Staining intensity of the MIPs in endometrial cancer cells were compared to the following three difference cellular compartments of the normal fertile control samples; Glandular epithelium, luminal epithelium and stroma (See Figure 14 below). We also made observations as to if there were differential staining in the nuclear and cytoplasmic compartments of the endometrial cells for the four MIPs and if there was differential blood vessel associated staining.

The endometrial samples obtained from healthy, normal postmenopausal women contained very thin, sparse amount of intact endometrium. Therefore the Quick Score method of analysis (which require analyzing 10 high power fields) was not appropriate or feasible. Postmenopausal samples were consequently analyzed using intensity scoring with descriptive localisation remarks for all three compartments, glandular epithelium, luminal epithelium and stroma.

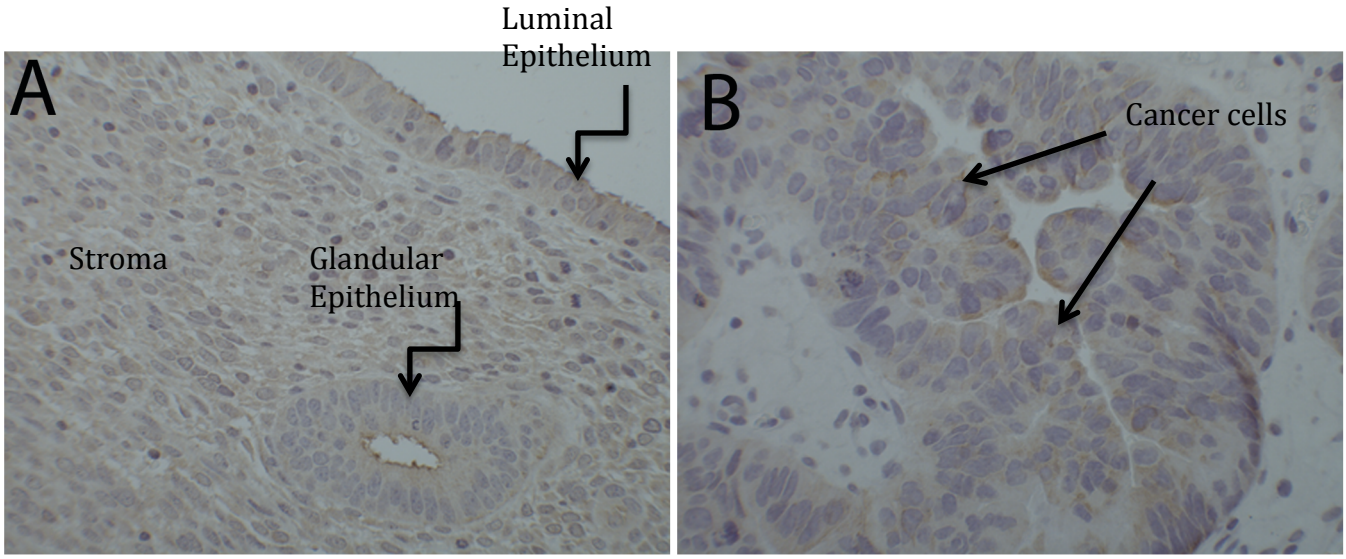


Figure 14: Demonstrating the different cellular compartments in a normal fertile control sample (A) and endometrial cancer cells in a endometrial cancer sample (B). (40x magnification)

4.2.a OSTEOPONTIN

In the normal fertile control OPN was primarily localised in the epithelium and in the blood vessels. In the epithelial compartment cytoplasmic staining was observed in both glandular and luminal epithelial cells. Moderate staining was seen in the vessels as seen in Fig 15.

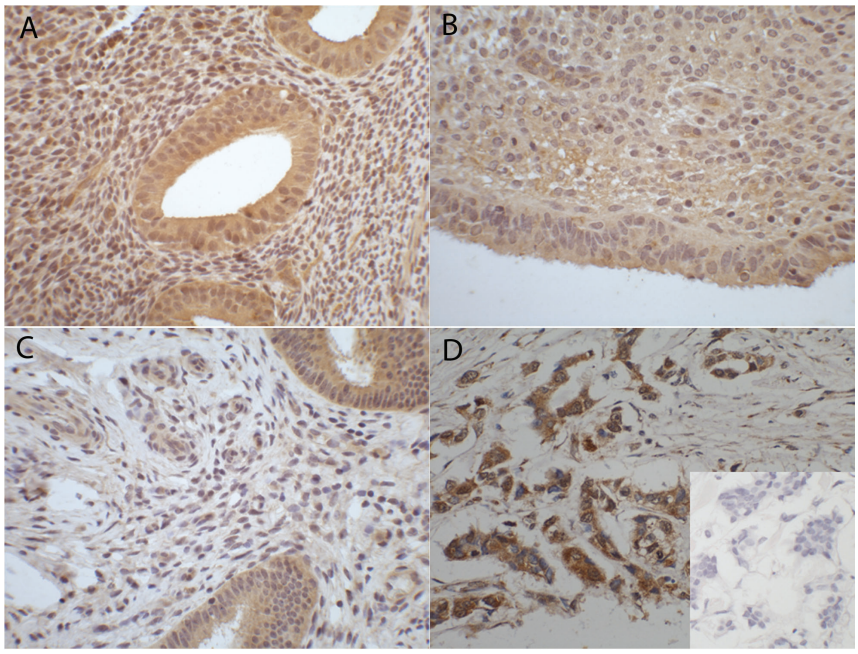


Figure 15: OPN IHC: Demonstrating moderate immuno-staining in the different cellular compartments. A: Glandular epithelium, B: Luminal epithelium, C: Vessels, D: Positive/Negative control. {40xmagnification}

OPN immuno-staining was primarily localised in the cytoplasm of glandular epithelial cells in the postmenopausal endometrium (See Fig 16). There is distinct and statistically significant difference observed between the OPN expression in normal fertile and postmenopausal glandular epithelial cells (Mann Whitney U test $p < 0.0001$).

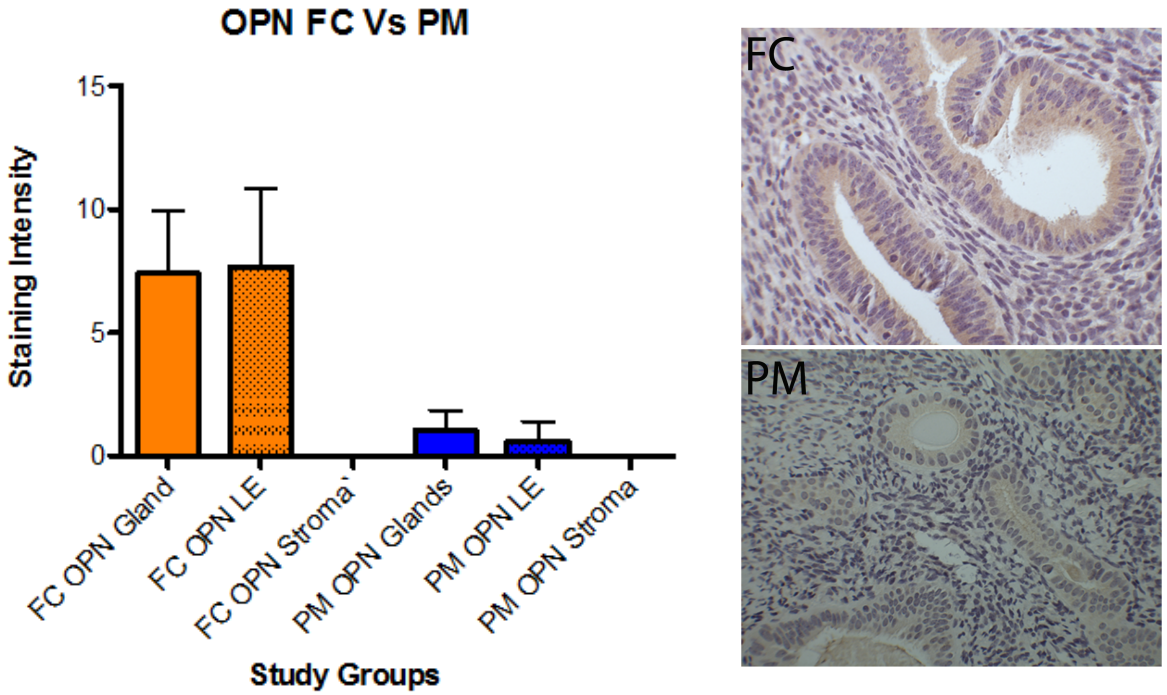


Figure 16: Comparison between OPN immuno-staining seen in glandular epithelium of normal fertile (FC) and postmenopausal (PM) endometrium. {40xmagnification} Results are represented as mean ± SD, FC n=15, PM n=10

Both cytoplasmic and nuclear staining was seen in the endometrial cancer cells as seen in Figures 17 and 18. Results comparing staining intensity of endometrial cancer cells and intensity in stromal compartment of normal fertile controls would be skewed since OPN is not normally present in the stroma. Therefore comparison between glandular and luminal epithelial cellular compartments with endometrial cancer cells will only be discussed.

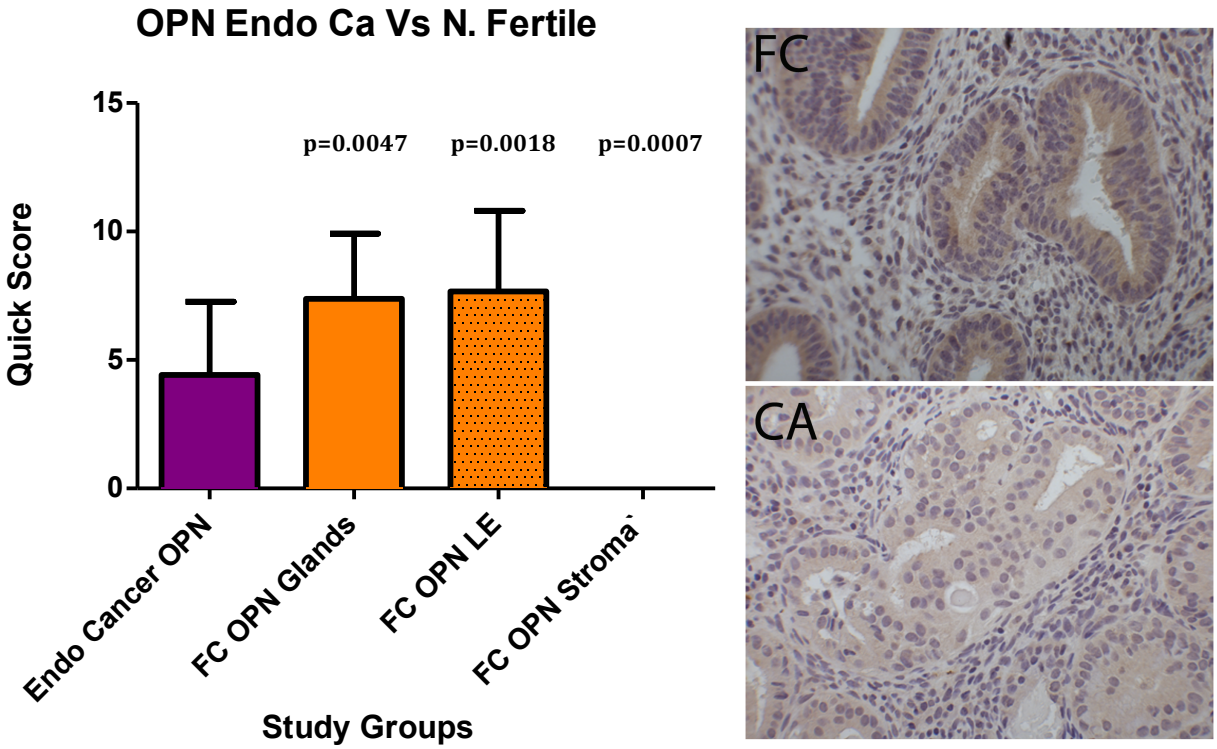


Figure 17: Comparison between OPN immuno-staining seen in endometrial cancer cells (CA) and normal fertile control (FC) {40xmagnifiaction} Results are represented as mean ± SD, FC n=15, CA n=30

Compared to the proliferative phase normal control endometrium, endometrial cancer cells showed decreased OPN immuno-staining seen in Figure 17. This difference was significant between the expression of OPN in endometrial cancer cells and glandular (Mann Whitney U test; p=0.0047) and luminal epithelial cells (Mann Whitney U test; p=0.0018). Therefore, endometrial cancer cells loose the expression of OPN compared to normal epithelial cells in the proliferative phase.

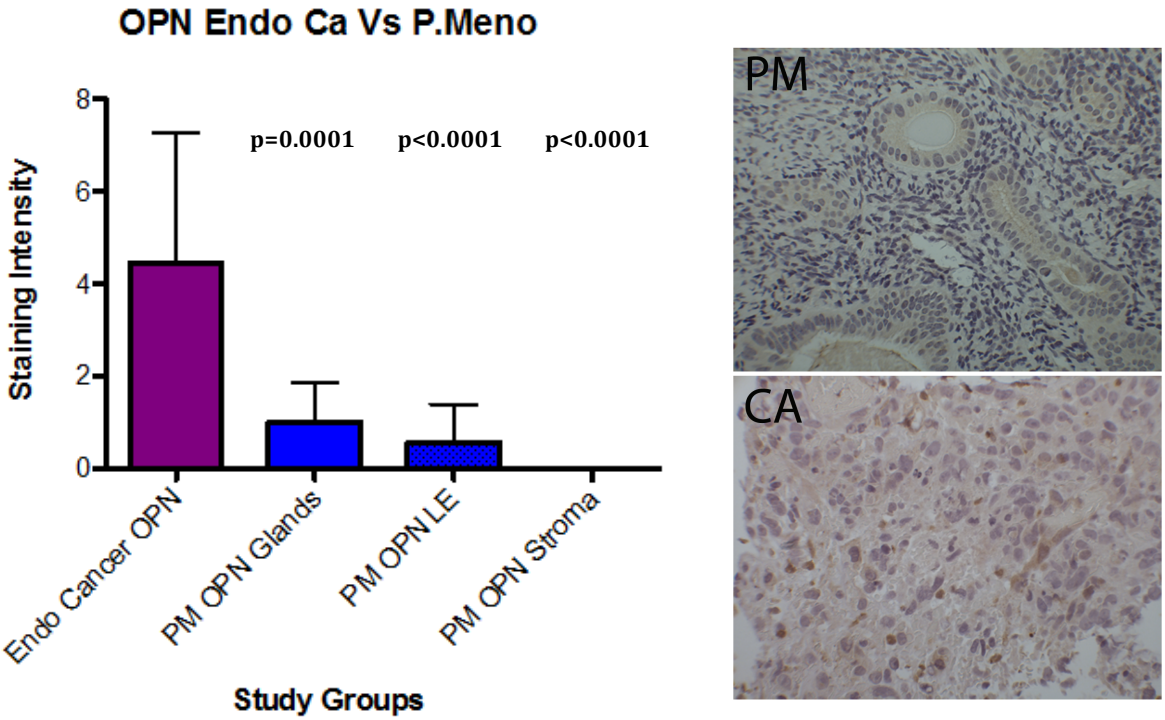


Figure 18: Comparison between OPN immuno-staining seen in endometrial cancer cells (CA) and postmenopausal endometrium (PM). {40xmagnification} Results are represented as mean ± SD, PM n=10, CA n=30.

When comparing immuno-staining between postmenopausal endometrium and endometrial cancer cell, results suggest endometrial cancer cells to have higher immuno-reactivity to OPN than postmenopausal endometrium. See Fig 18.

There was a statistically significant difference seen between the immuno-staining of OPN in endometrial cancer cells and all cellular compartments in postmenopausal endometrium.

No correlation was observed between osteopontin immuno-staining and FIGO grade of all the endometrioid endometrial cancers in the sample set of the study (Pearson's correlation r = 0.3027, p = 0.1709) as seen in Fig 19.

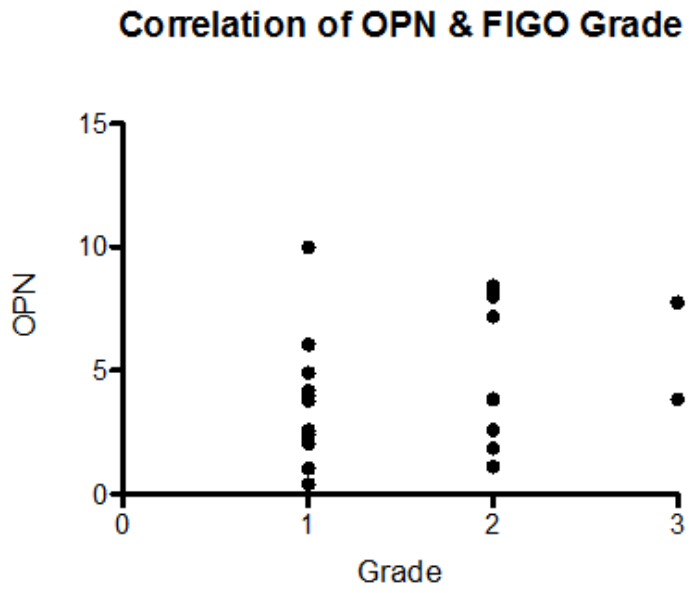


Figure 19: Correlation graph showing the relationship between OPN and FIGO Grade of endometrioid endometrial cancer

4.2.b S100P

Endometrial immuno-reactivity for S100P was largely confined to the stromal compartment in the proliferative phase control group, but occasionally a few positive cells were seen in the glandular and luminal epithelium. Both nuclear and cytoplasmic staining was observed. Blood vessels were also moderately stained in a few of the sample where the staining was seen in the below, Fig 20.

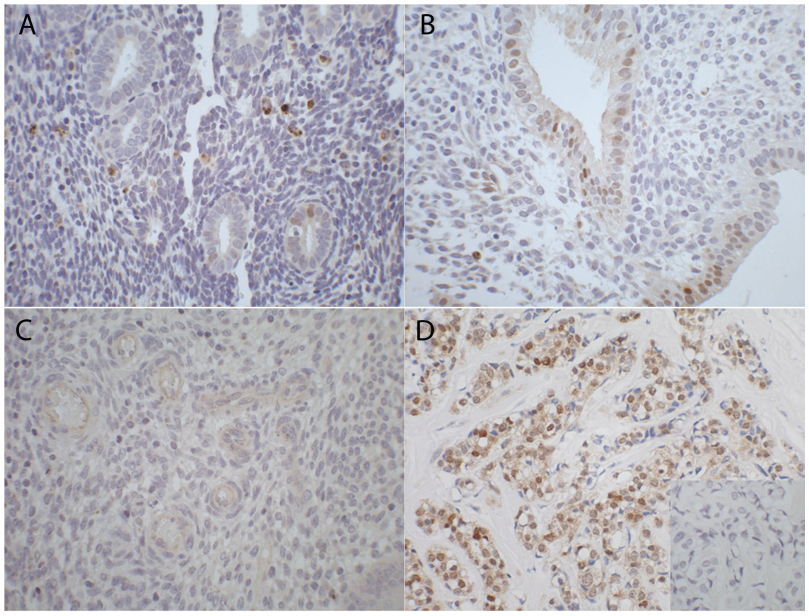


Figure 20: IHC for S100P; A: Stromal stain, B: Glandular and luminal epithelial, C: Vascular, D: Positive and negative control. {40xmagnification)

Weak-moderate staining was seen in the glandular epithelial compartment in postmenopausal endometrium, localised to the cytoplasm of the cells (Fig 21). There was a statistically significant difference observed between glandular epithelial immuno-stain between the two control groups (Mann Whitney U test; p=0.0207).

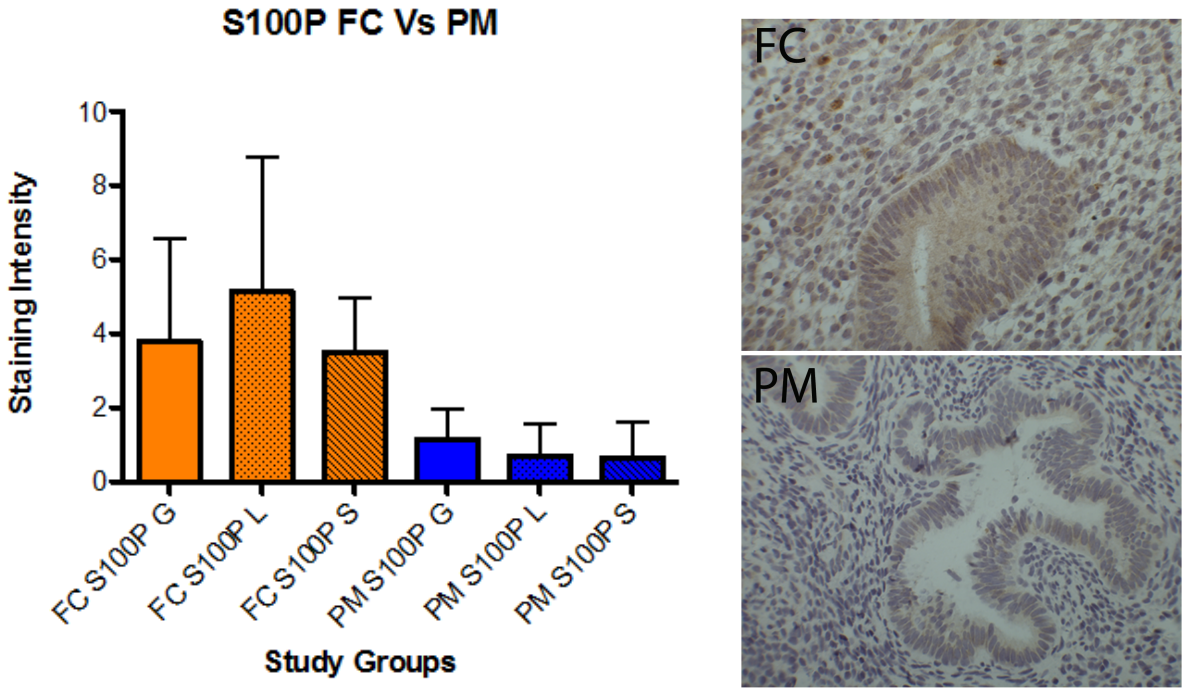


Figure 21: Comparison of IHC on normal fertile (FC) and postmenopausal (PM) glandular epithelium. {40xmagnification} Results are represented as mean ± SD, FC n=15, PM n=10

Both cytoplasmic and nuclear staining was observed in the endometrial cancer cells (Fig 22 and 23). Our results show apparently increased immuno-staining for S100P in endometrial cancer cells when compared to all cellular compartments in normal fertile control (Fig 23). However, this differential staining of the endometrial cancer cells was statistical significant only when compared with the stromal compartment (Mann Whitney U test; $p < 0.0221$).

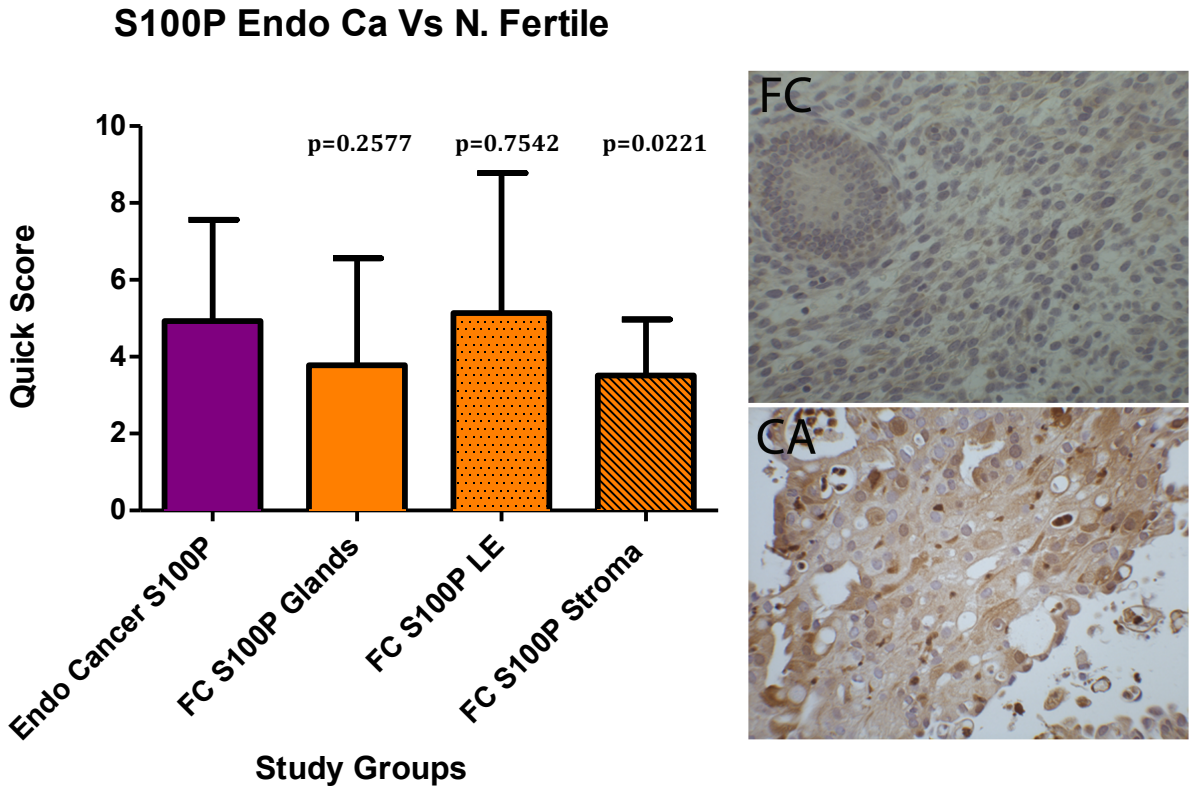


Figure 22: IHC Comparison between of S100P normal fertile (FC) and endometrial cancer (CA). {40xmagnification} Results are represented as mean ± SD, FC n=15, CA n=30

S100P immuno-reactivity was not observed in the normal postmenopausal endometrium compared to endometrial cancer cells (Fig 23). The difference observed between the two groups was statistically significant, with an increased immuno-reactivity to S100P seen in endometrial cancer cells.

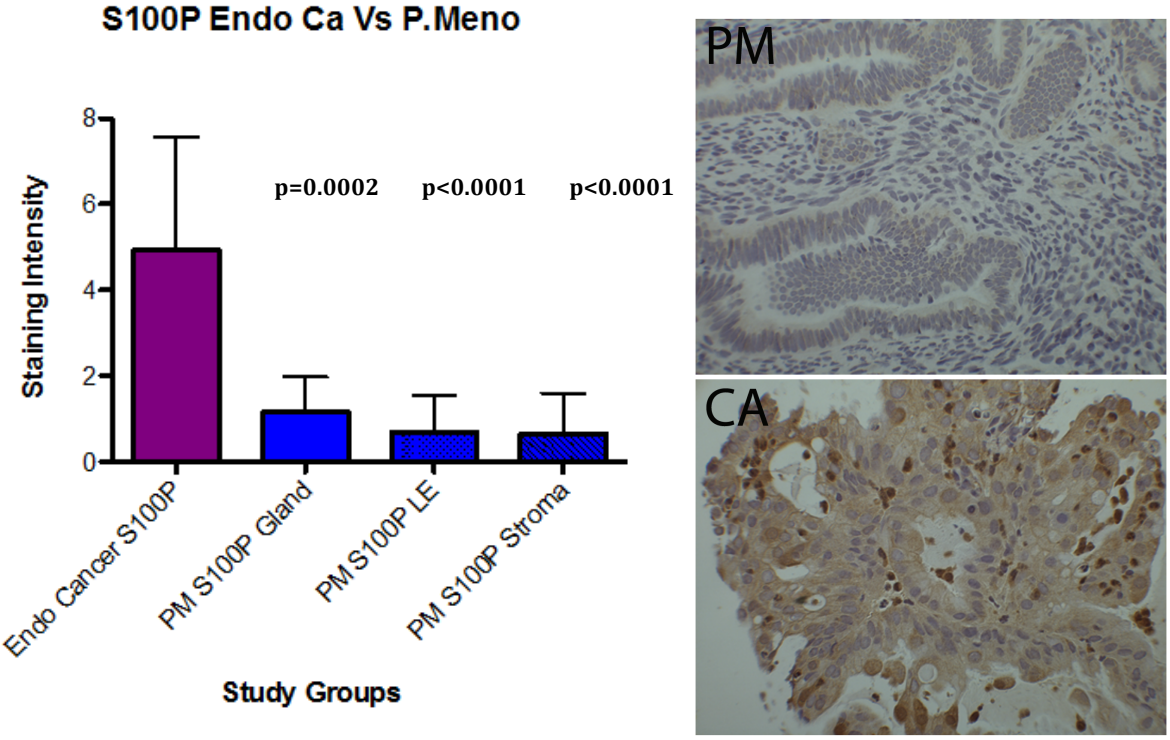


Figure 23: IHC comparison between endometrial cancer cells (CA) and postmenopausal endometrium (PM). {40xmagnification) Results are represented as mean ± SD, PM n=10, CA n=30

There was no association seen between S100P expression and the different FIGO grades of endometrioid endometrial cancers (Pearson's correlation, r = 0.3700, p = 0.0901). As shown below in Fig 24:

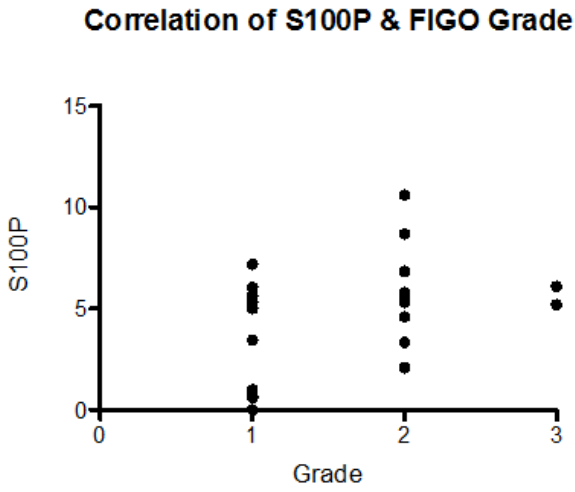


Figure 24: Graph showing the correlation between S100P and FIGO grade of endometrioid endometrial cancer

4.2.c AGR-2

Normal fertile control samples showed immuno-staining for AGR2 in the glandular and luminal epithelium, where the immuno-staining was seen in the epithelial cell cytoplasm. No vascular staining was observed in the normal fertile control samples (Fig 25).

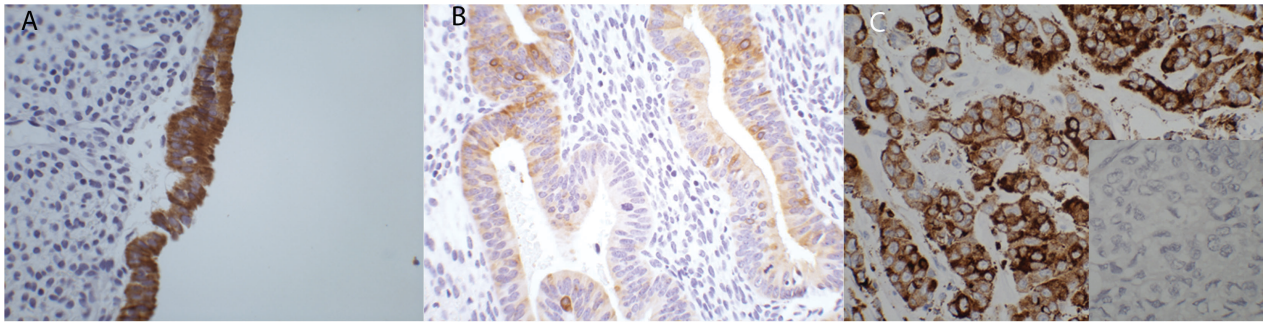


Figure 25: IHC showing AGR2 immuno-stain in different cellular compartments. A: Luminal epithelium, B: Glandular epithelium, C: Positive/negative control. {40xmagnifiaction}

Normal postmenopausal samples showed immuno-staining for AGR2 expression in the cytoplasm of glandular and luminal epithelial compartments (Fig 26). In comparison to the normal fertile endometrium, a significant difference was seen in the expression of AGR2, with reduced immuno-stain observed in the postmenopausal samples (Mann Whitney U test; Glandular epithelium $p < 0.0001$, Luminal epithelium $p < 0.0003$).

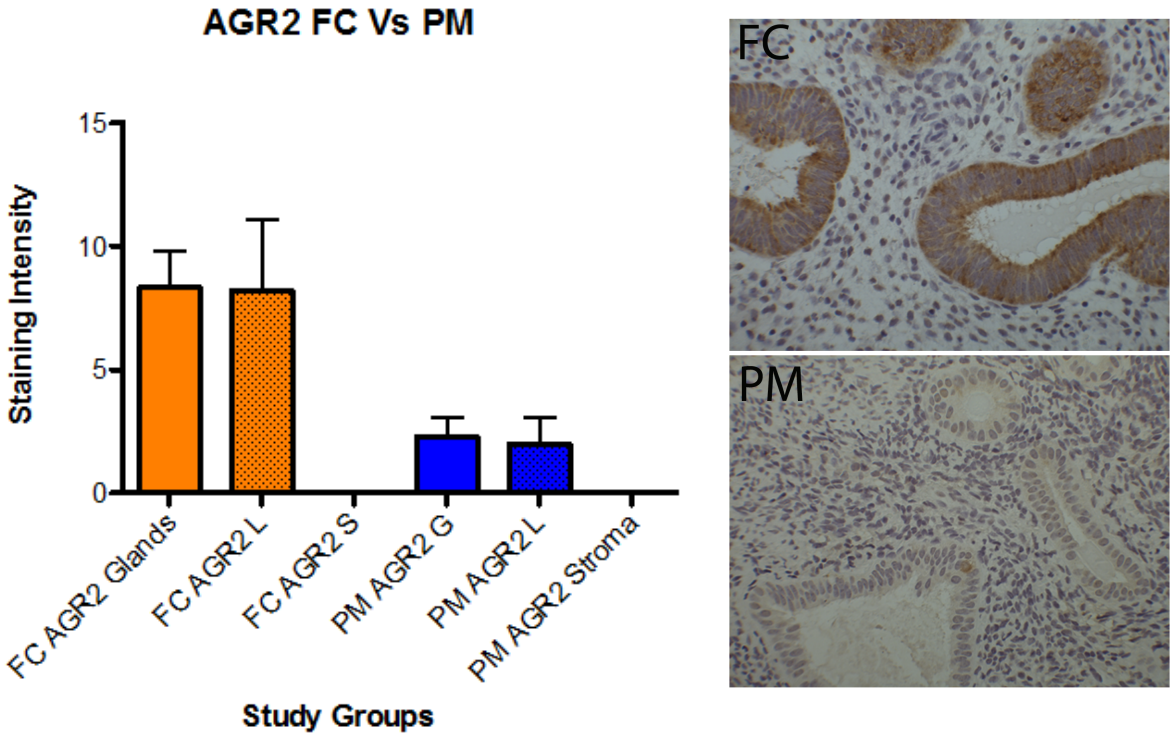


Figure 26: Comparison between normal fertile (FC) and postmenopausal (PM) AGR2 immuno-stain. {40xmagnification} Results are represented as mean ± SD, FC n=15, PM n=10

Positively stained endometrial cancer cells showed immune-reactivity for AGR2 both in the nucleus and in the cytoplasm (Figure 27 and 28). Results comparing staining intensity of endometrial cancer cells and intensity in stromal compartment of normal fertile controls would be skewed since AGR2 is not normally present in the stroma. Therefore comparison between glandular and luminal epithelial cellular compartments with endometrial cancer cells will only be discussed.

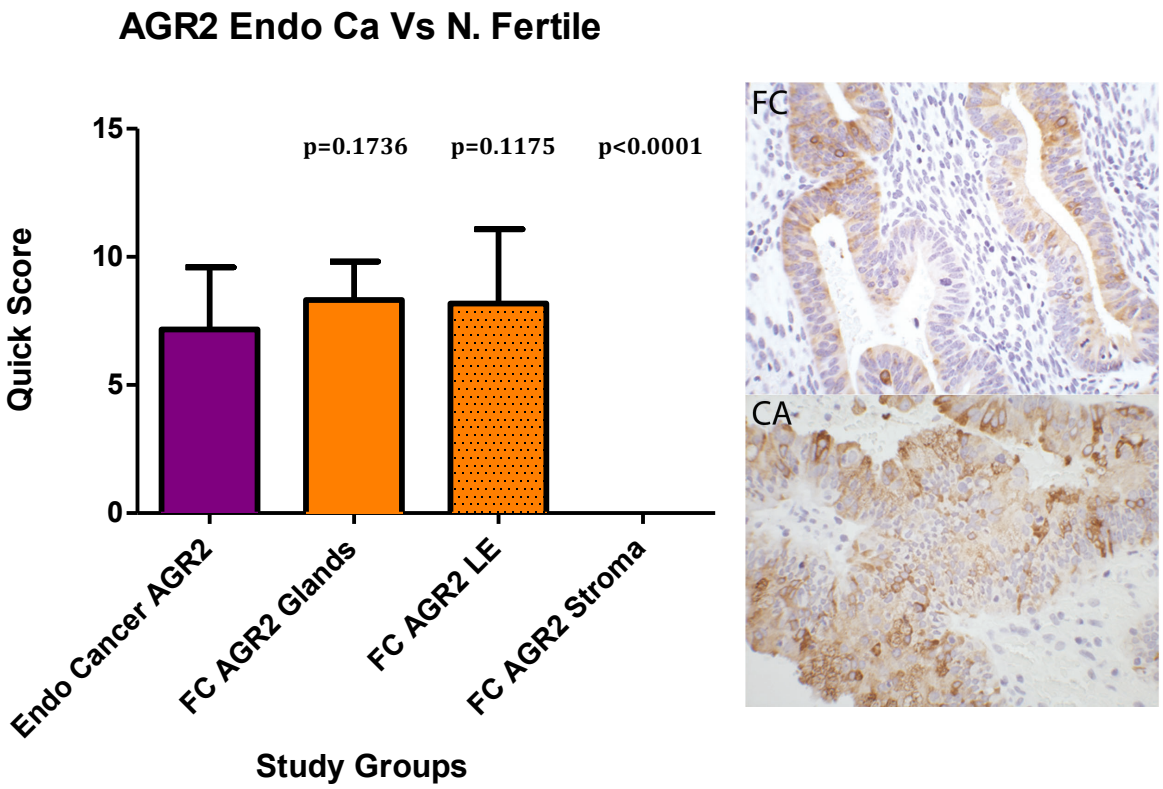


Figure 27: Comparison between AGR2 immuno-stain on endometrial cancer cells (CA) and normal fertile (FC) endometrium. {40xmagnification} Results are represented as mean ± SD, FC n=15, CA n=30

Results generated suggest that endometrial cancer cells express less AGR2 compared to normal fertile epithelial cells (Fig 27). However, semi-quantitative comparison of immuno-staining for AGR2 between all endometrial cancer cells and the fertile controls do not show a statistically significant difference (Mann Whitney U test; Glandular epithelium $p < 0.1736$, Luminal epithelium $p < 0.1175$).

However, in comparison to postmenopausal endometrium, endometrial cancer cells have been shown to over express AGR2 (Fig 28).

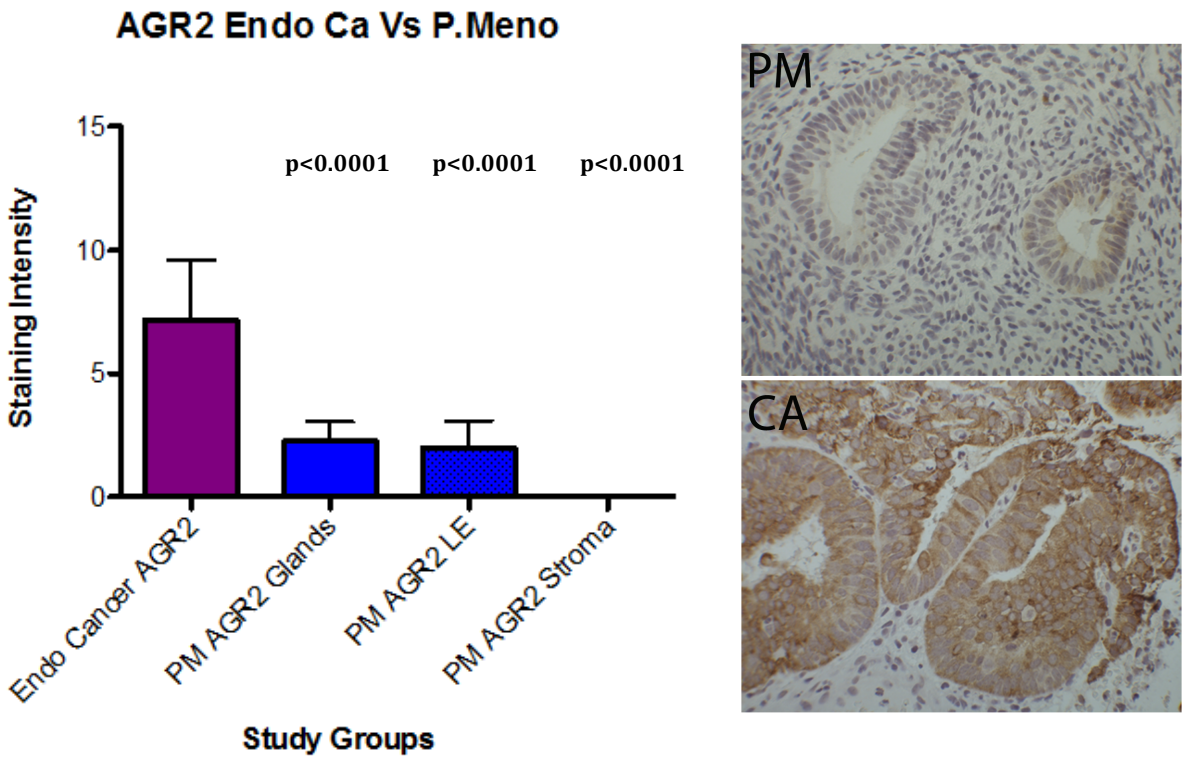


Figure 28: IHC comparing immuno-staining of AGR2 between endometrial cancer cell and postmenopausal endometrium. {40xmagnifiaction} Results are represented as mean ± SD, PM n=10, CA n=30.

The results showed a significant difference between the two groups, demonstrating an increased immuno-reactivity to AGR2 in endometrial cancer cells.

No correlation was observed between immuno-staining of AGR2 in endometrial cancer cells and their FIGO grading (Pearson correlation r = -0.3689, p = 0.0911). See Figure 29 below:

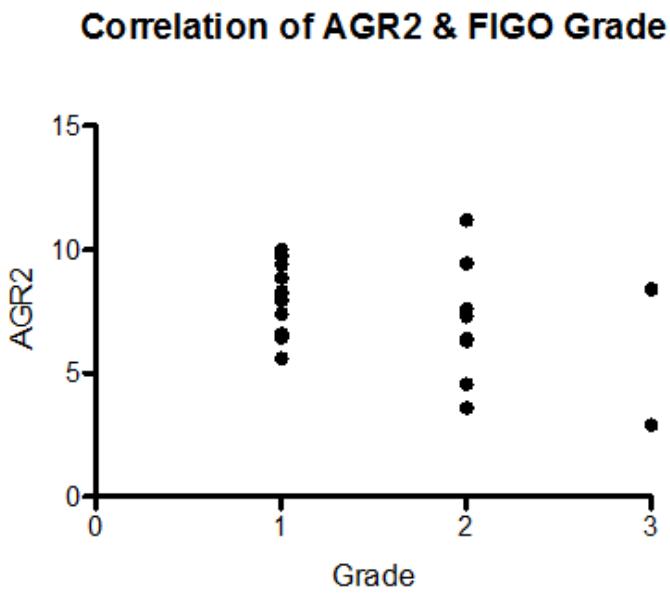


Figure 29: Graph showing the correlation between AGR2 and FIGO grade of endometrioid endometrial cancer

4.2.d S100A4

Positive immuno-staining was observed predominantly in the stromal compartment of normal fertile control samples, localised to both to the cytoplasm and nucleus. There were occasional (<1 in 50 cells) nuclear and cytoplasmic epithelial cell S100A4 staining seen. Moderate S100A4 immuno-staining was also localized to blood vessels of normal fertile control endometrium (Fig 30).

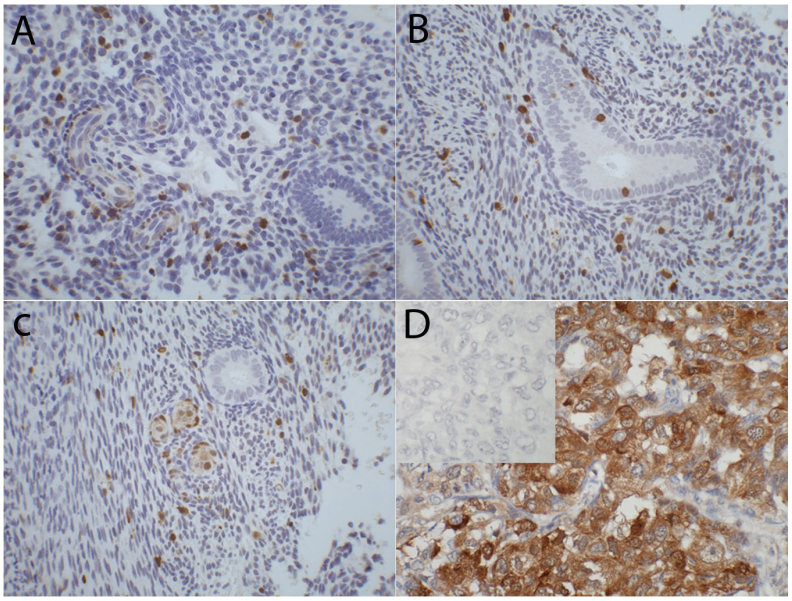


Figure 30: S100A4 staining observed in epithelial, stromal and vascular compartments. A: Stromal, B: Epithelial, C: Vessels, D: Positive/negative control. {40xmagnification}

Moderate staining of S100A4 was seen in all cellular compartments in the normal postmenopausal endometrium. Immuno-staining was localised to the nucleus and cytoplasm of epithelial and stromal cells (Fig 31). A statistically significant difference was only generated between the normal fertile and postmenopausal endometrium in the stromal and glandular epithelial cellular compartments (Mann Whitney U test; Stromal $p = 0.0034$, Glandular epithelium $p < 0.0001$). Stromal S100A4 immuno-reactivity was higher in normal fertile endometrium, and glandular staining was lower in comparison to postmenopausal endometrium.

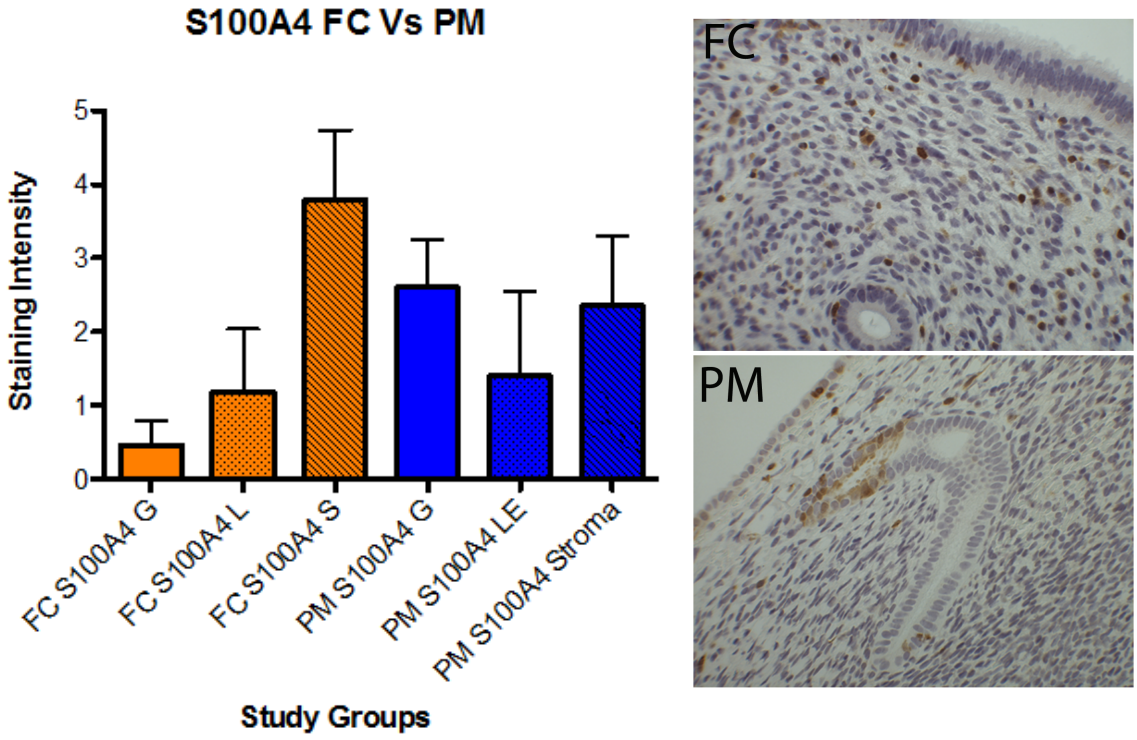


Figure 31: Comparison between normal fertile (NF) and postmenopausal (PM) endometrium for S100A4 staining. {40xmagnification} Results are represented as mean ± SD, FC n=15, PM n=10

Cytoplasmic S100A4 staining was observed in positive endometrial cancer cells (Fig 32 and 33). In comparison to normal fertile endometrium, a statistical difference was seen, demonstrating the over expression of S100A4 in endometrial cancer cells (Fig 32). All three normal fertile endometrial cell compartments studied showed a statistically significant difference compared with endometrial cancer cells.

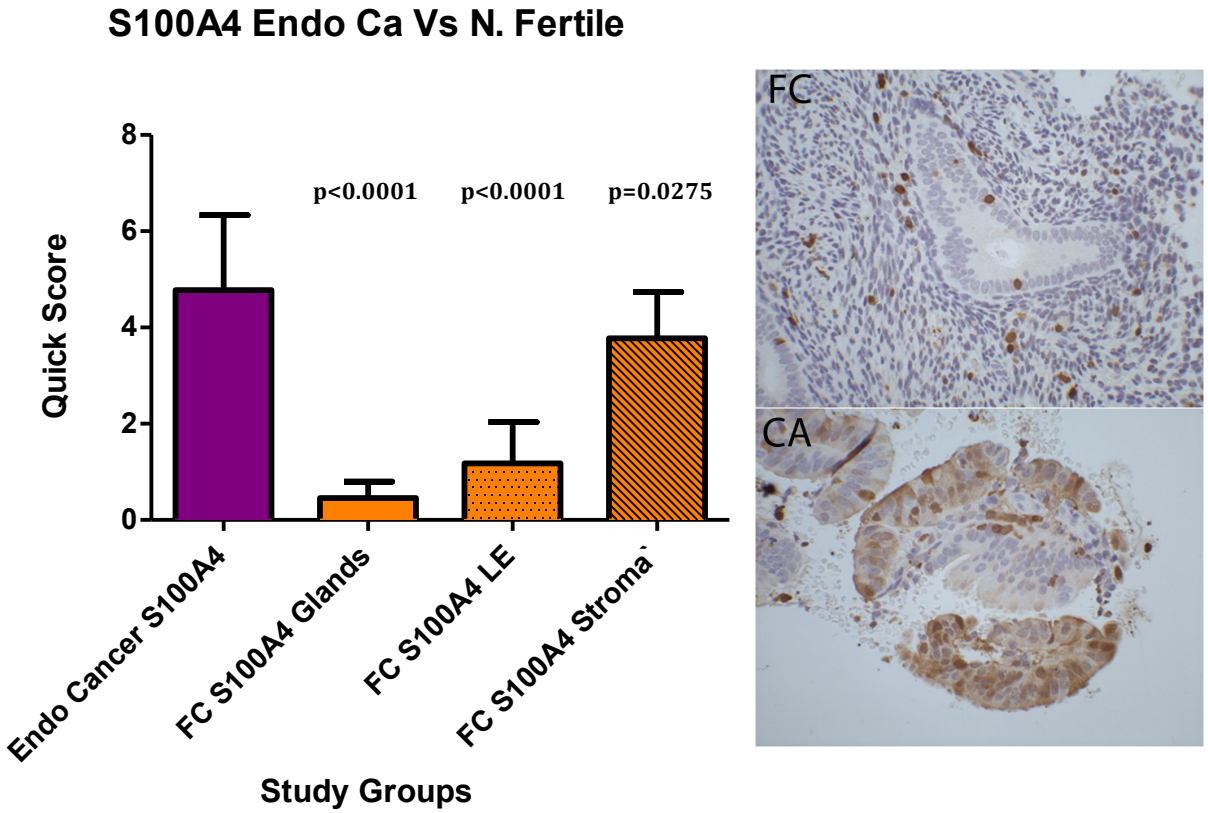


Figure 32: Comparing immuno-stain of S100A4 between endometrial cancer cells (CA) and normal fertile (FC) endometrium. {40xmagnification} Results are represented as mean ± SD, FC n=15, CA n=30

The results also showed increased immuno-reactivity for S100A4 in endometrial cancer cells in comparison to normal postmenopausal endometrium (Fig 33). The difference observed between the three cellular compartments was statistically significant.

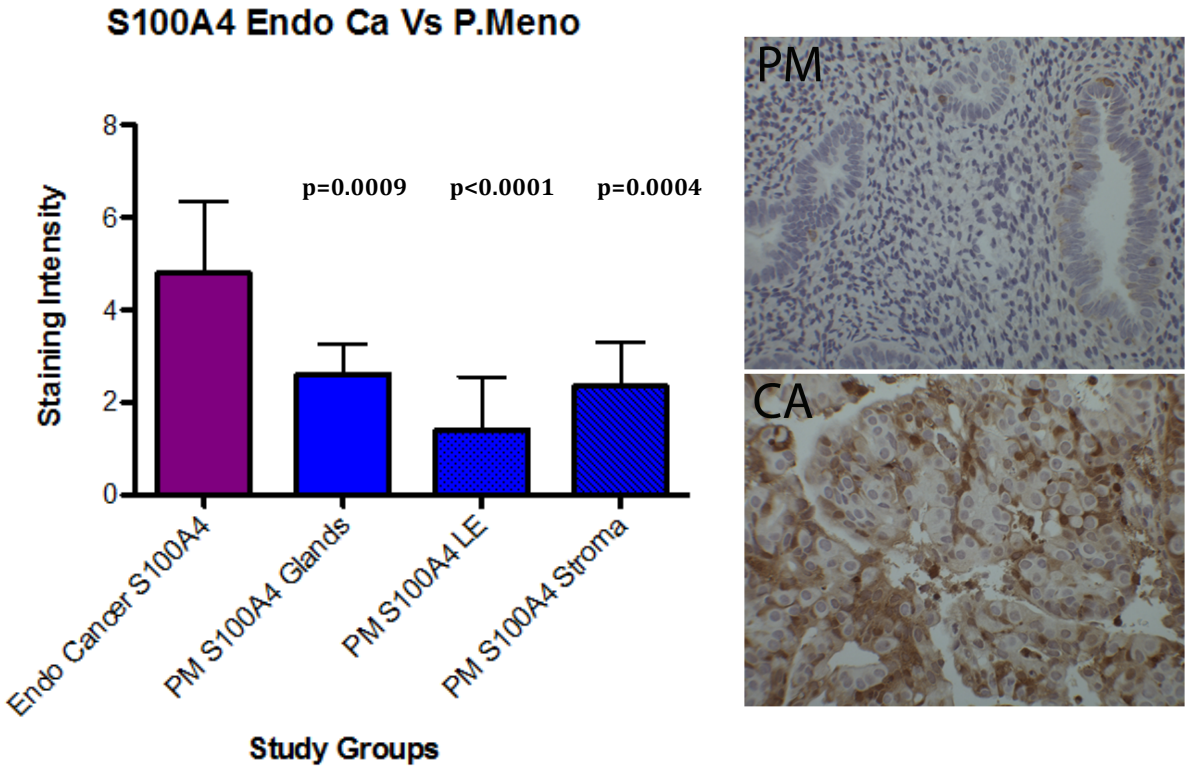


Figure 33: IHC results comparing S100A4 staining between endometrial cancer cells (CA) and postmenopausal endometrium (PM) {40xmagnification} Results are represented as mean ± SD, PM n=10, CA n=30

There was no correlation seen between endometrial cancer grade and expression of S100A4 (Pearson correlation r = -0.3339, p = 0.1288). See Fig 34 below:

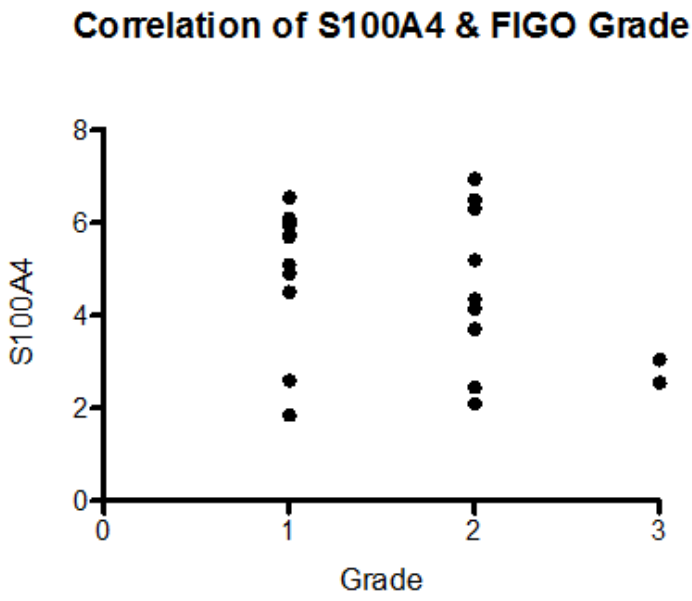


Figure 34: Correlation graph showing the relationship between S100A4 immuno-stain and FIGO Grade

4.3 RT-PCR

The occurrence of the MIPs mRNA in human benign endometrial tissue and malignant endometrial carcinomas were examined by revere transcription- PCR. Band intensities for each sample were obtained using Image J software and normalized with its corresponding GAPDH band (Fig 35, Tables 19 & 20).

Mann Whitney U test could not be carried out for the direct comparison of normal fertile and endometrial cancer sample, as a minimum of three samples in each group is required. Therefore Kruskal-Wallis statistical test was perfomed comparing all three groups (normal fertile control, endometriosis and endometrial cancer).

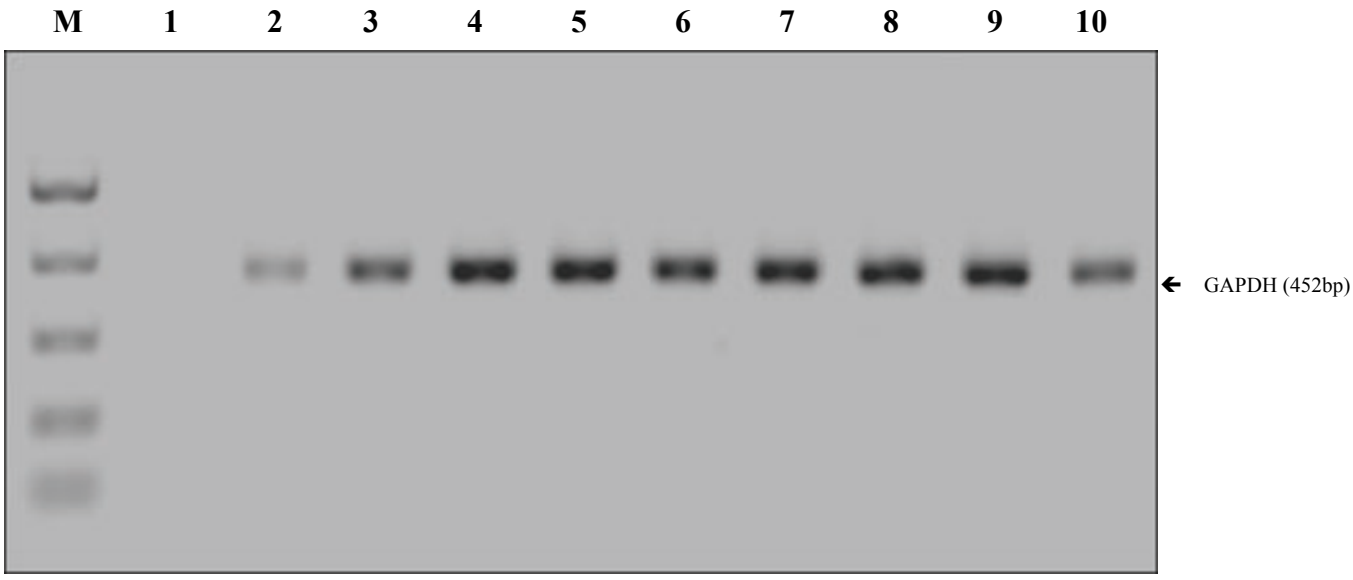


Figure 35: GAPDH normalization control for samples (L-V) Lane M: DNA Ladder, Lane 1: Negative control, 2: Positive control, 3-5: Normal fertile, 6-8: Endometriosis, 9 &10: Endometrial Cancer.

Table 19: Average and normalized values for OPN and S100P mRNA samples

Samples	OPN		S100P	
	Average	Normalized	Average	Normalized
Endometriosis	11928	11928	7628	7628
Endometriosis	17336	10507	7741	4692
Endometriosis	17194	10473	15721	9576
Normal	6643	4939	671	499
Normal	3575	2328	282	184
Normal	4192	2656	639	405
Endo Ca	16855	10385	12192	7512
Endo Ca	14863	15503	1183	1234

Results suggest that endometrial cancer cells over express Osteopontin (KW test; $p = 0.0821$), S100P (KW test; $p = 0.0622$) and S100A4 (KW test; $p = 0.0622$) mRNA in comparison to normal fertile endometrium. However, no statistically significance was observed. (See figures 36 and 37 for OPN, 38 and 39 for S100P & 42 and 43 for S100A4 results).

Table 20: Average and normalized values for AGR2 and S100A4 mRNA samples

Samples	AGR2		S100A4	
	Average	Normalized	Average	Normalized
Endometriosis	6030	6030	4774	4774
Endometriosis	7879	4775	7962	4826
Endometriosis	10036	6113	10183	6202
Normal	9190	6833	4286	3187
Normal	4392	2860	3714	2418
Normal	4573	2897	3314	2099
Endo Ca	2203	1357	5301	3266
Endo Ca	4330	4517	2853	2975

Likewise, no significant difference was observed for AGR2 mRNA expression between the endometrial cancer and normal fertile control samples (KW test; $p = 0.2905$). Results suggest there to be an apparent reduction of AGR2 expression in endometrial cancer cells. See figures 40 & 41.

4.3.a OSTEOPONTIN

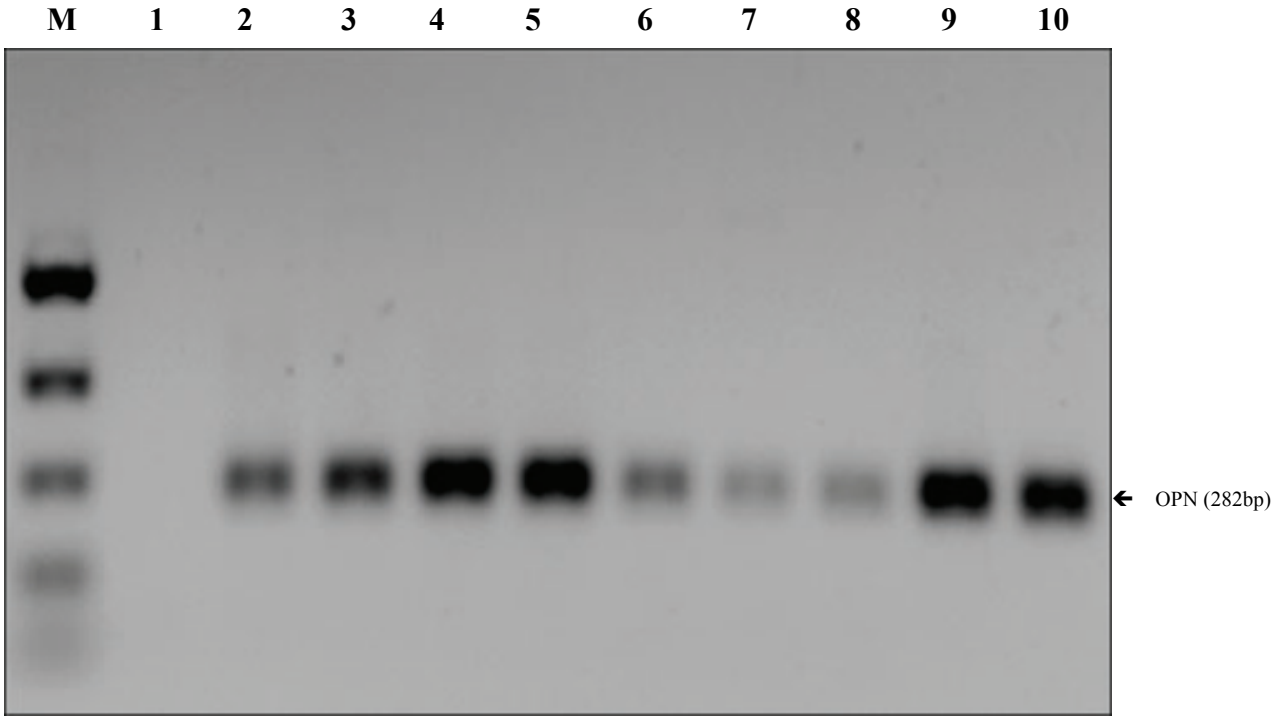


Figure 36: OPNmRNA expression for samples (L-V) Lane M: DNA Ladder, Lane 1: Negative control, 2: Positive control, 3-5: Normal fertile, 6-8: Endometriosis, 9 &10: Endometrial Cancer.

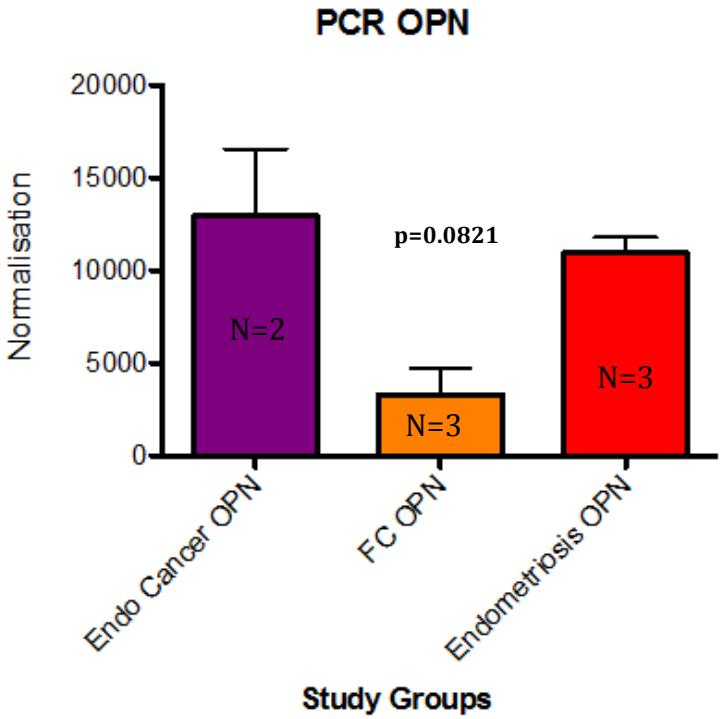


Figure 37: Box plot comparing OPN mRNA expression between Endo cancer, normal fertile and endometriosis samples. Results are represented as mean ± SD.

4.3.b S100P

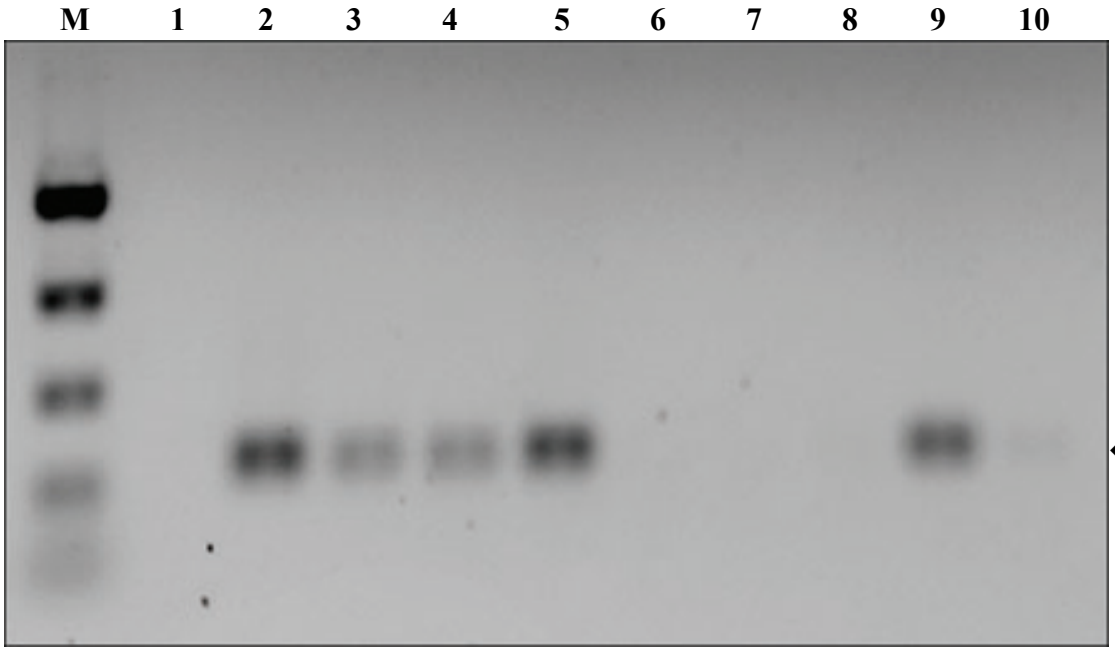


Figure 38: S100PmRNA expression for samples (L-V) Lane M: DNA Ladder, Lane 1: Negative control, 2: Positive control, 3-5: Normal fertile, 6-8: Endometriosis, 9 &10: Endometrial Cancer.

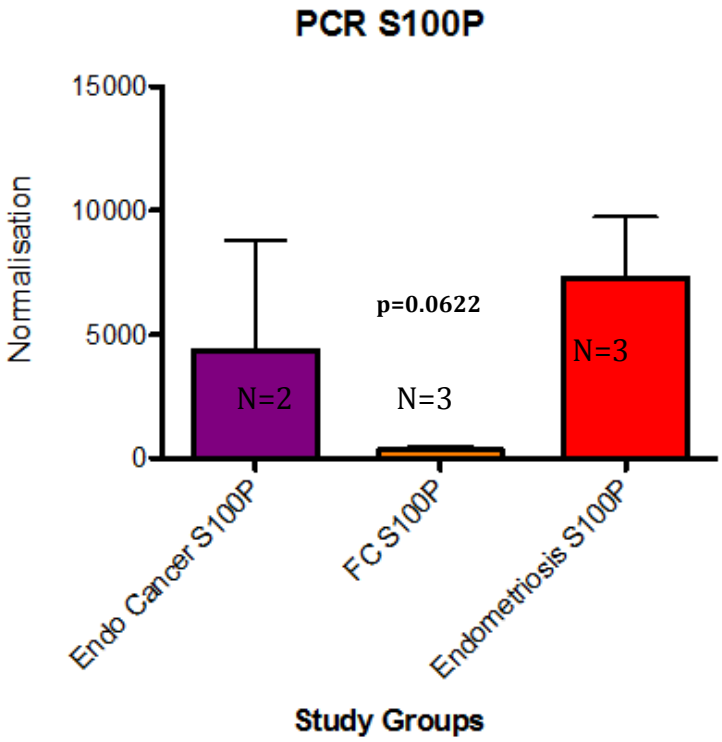


Figure 39: Box plot comparing S100P mRNA expression between Endo cancer, normal fertile and endometriosis samples. Results are represented as mean ± SD.

4.3.c AGR2

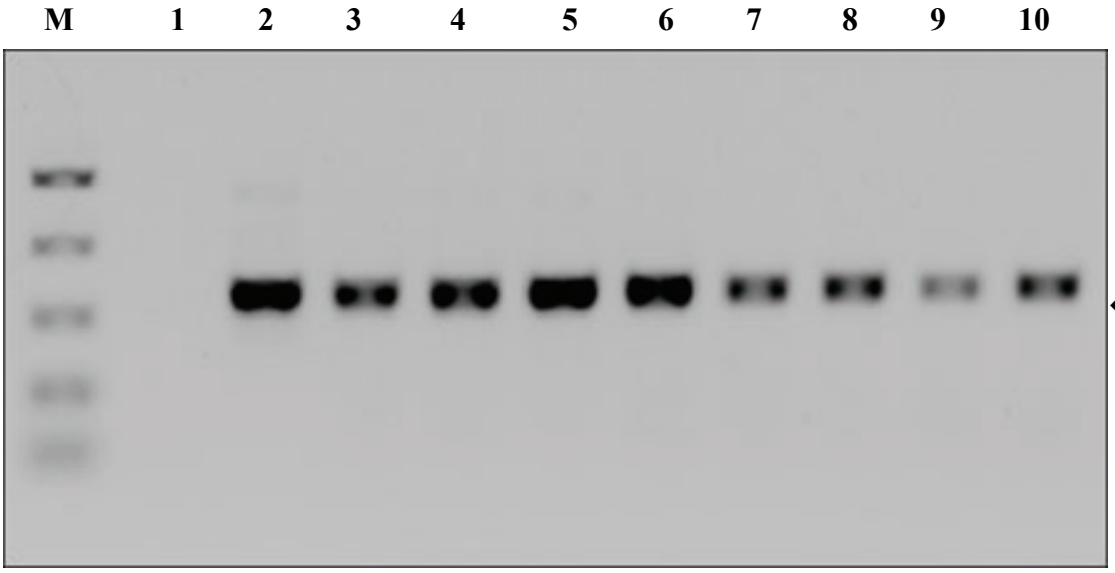


Figure 40: AGR2mRNA expression for samples (L-V) Lane M: DNA Ladder, Lane 1: Negative control, 2: Positive control, 3-5: Normal fertile, 6-8: Endometriosis, 9 &10: Endometrial Cancer.

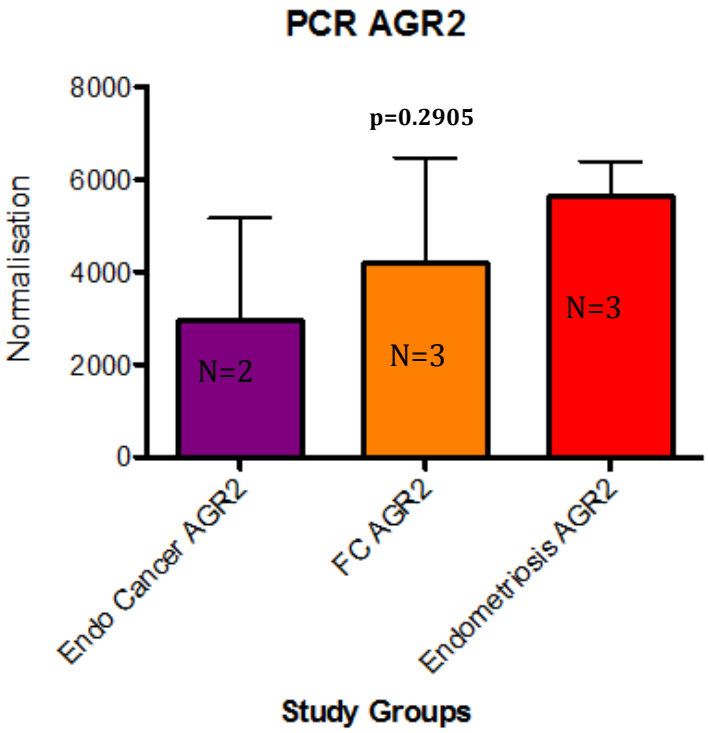


Figure 41: Box plot comparing AGR2 mRNA expression between Endo cancer, normal fertile and endometriosis samples. Results are represented as mean ± SD.

4.3.d S100A4

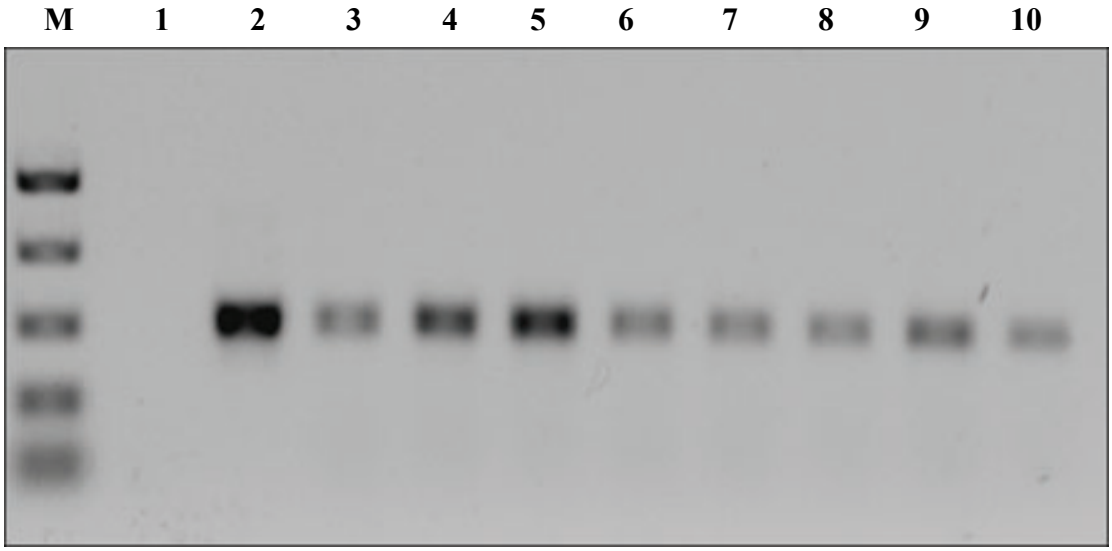


Figure 42: S100A4mRNA expression for samples (L-V) Lane M: DNA Ladder, Lane 1: Negative control, 2: Positive control, 3-5: Normal fertile, 6-8: Endometriosis, 9 &10: Endometrial Cancer.

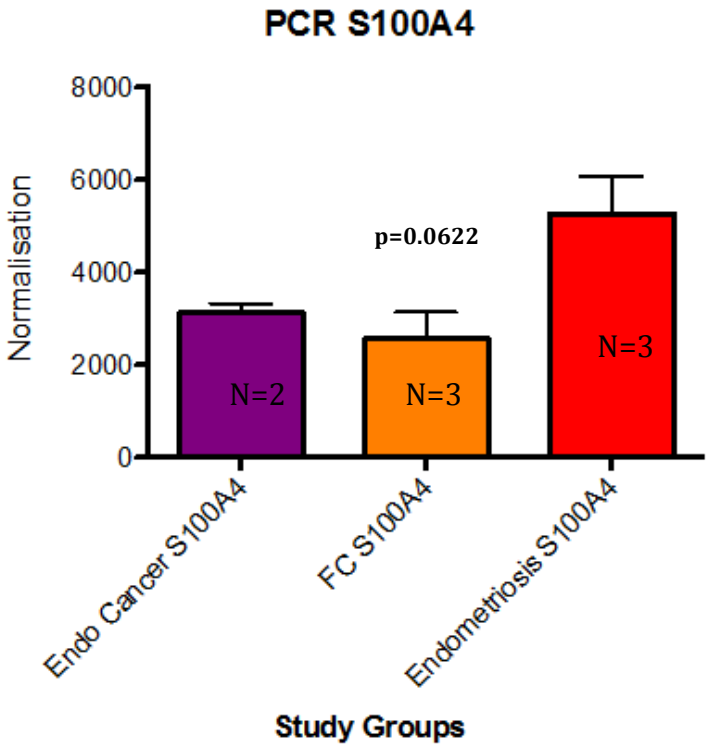


Figure 43: Box plot comparing OPN mRNA expression between Endo cancer, normal fertile and endometriosis samples. Results are represented as mean ± SD.

Overall results from RT-PCR have confirmed IHC results for the expression of S100P, AGR2 and S100A4, however OPN results were contradictory.

5 DISCUSSION

The aim of this pilot study was to examine the expression of Osteopontin, S100P, AGR2 and S100A4 in human endometrial cancer cells. We also included normal fertile and postmenopausal endometrium as control groups to detect any aberrant expression of the MIPs specific to the endometrial cancer samples. All four metastasis inducing proteins were located in the endometria from normal fertile and postmenopausal women using immunohistochemistry. Results showed increased immuno-reactivity for S100P and S100A4 in endometrial cancer cells, but reduced immuno-reactivity was observed for OPN and AGR2. Similar results were also generated using reverse transcriptase polymerase chain reaction, for S100P, S100A4 and AGR2 confirming the immunohistochemistry analysis. However RT-PCR results showed that endometrial cancer cells expressed higher levels of OPN compared to normal fertile control samples, which is in contrast to our IHC results. These results will be discussed in detail in the following chapter.

5.1 NORMAL FERTILE ENDOMETRIUM

Endometrial staining results observed in normal fertile proliferative phase endometrium for all four MIPs were consistent with previous studies (Hapangama et al., 2011 (In press)).

5.1.a OSTEOPONTIN

Osteopontin is expressed by epithelial cells in primate and human endometrium (Allan et al 2003;Lessey 2002; Odagiri et al 2007). Studies have shown that OPN is a progesterone regulated endometrial secretory protein (Johnson et al 2003). OPN has been shown to be detectable in both the proliferative and early secretory phase endometrium in the glandular and luminal epithelium (Apparao et al 2001).

Moderate immune-reactivity for OPN was seen in the proliferative phase normal fertile endometrial sample in this study, disagreeing with some previous studies but agreeing with others (Apparao et al 2001). Staining was localized to cytoplasm of the glandular and luminal epithelial cellular compartments. The difference observed could be due discrepancies in OPN antibodies and IHC methods used. In previous work done in our laboratory, a different OPN antibody (mouse monoclonal anti-human OPN,) gave a much lower intensity of immuno-staining compared to the rabbit polyclonal anti-human OPN I used for my study. Study done by Apparao et al (2001) used monoclonal mouse anti-human OPN antibody without conducting antigen retrieval step and we carried out high temperature antigen retrieval, which is thought to enhance the immuno-staining.

5.1.b S100P

S100P function in the normal endometrium is yet to be determined, but studies have suggested that it may play a role in the implantation window (Tong et al 2010; Hapangama DK et al., 2011 (In press)). The protein is primarily localised in both

epithelial and stromal compartments in normal fertile endometrium, with weak staining in proliferative phase endometrium (Hapangama DK et al., 2011 (In press)).

S100P mRNA levels were also shown to be at highest in the mid-late secretory phase, when systemic levels of progesterone are highest, suggesting S100P is a novel progesterone-regulated gene in the endometrium (Xie et al 2006). However, the progesterone regulation of S100P is uncertain. In contrast, in breast cancer specimens there was no correlation seen between S100P and oestrogen or progesterone receptor expression (Wang et al 2006). Further work is required to establish a link between progesterone and S100P.

S100P immuno-reactivity was localised to the stromal cellular compartment in the normal fertile endometrium in this study. There was a reduced immune-reactivity seen in the normal fertile endometrium compared to immuno-staining observed in endometrial cancer cells. S100P is known to have a proliferative role in cancers; therefore if there is a similar function in the endometrium, during the proliferative phase you would expect to find S100P expression. However, further functional studies in vitro need to be undertaken to ascertain this.

5.1.c AGR2

Human AGR2 is known to be strongly expressed in tissues containing mucus secreting cells and/or functioning as endocrine organs, this includes glandular epithelium in the endometrium (Brychtova et al 2011).

The first study reporting the expression of AGR2 in human endometrium arises from our laboratory (Hapangama et al 2011 (In press)). A cyclical variation in the expression of AGR2 in the normal fertile control samples was observed, with weakest

immuno-staining seen in the proliferative phase in all cellular compartments except endothelial cells. Increased immuno-reactivity of AGR2 was seen in endometriosis samples in the secretory phase. Analysis of AGR2 in my study showed positive expression in the glandular and luminal epithelium of normal fertile endometrium during the proliferative phase.

AGR2 is an oestrogen and androgen responsive protein, up regulated in a number of cancers, including breast, lung, ovarian, gastric, pancreatic, oesophageal, and prostate. Its expression in all premalignant and malignant oesophageal adenocarcinoma suggests that it serves an important role in disease pathogenesis (Wang et al 2008).

Further work is required to elicit the role it plays in the normal endometrium, which can be done by undertaking functional studies in vitro.

5.1.d S100A4

S100A4 is localised in the nucleus, cytoplasm, and the extracellular space. Cytoplasmic expression is evident in the vast majority of S100A4-expressing cells (Boye & Maelandsmo 2010). A study investigating expression of the MIPs in normal endometrium showed that S100A4 was immuno-localised to the endometrial stromal, perivascular and endothelial cells of normal fertile women (Hapangama et al 2011 (In press)).

Similar results were obtained from this study, where immuno-stain was primarily localised to the stromal compartment of the normal fertile control endometrium.

S100P and S100A4 belong to the S100 family of calcium-binding proteins, which have been shown to play a fundamental role in the metastatic phase of cancer (Wang

et al 2006; Grigorian et al 2008). Recent work has been reviewed, demonstrating S100A4-mediated effects on cell proliferation, apoptosis and tumor growth (Sherbet 2009). This may suggest that S100A4 has a proliferative function in normal endometrial cells, hence expressed in the proliferative phase.

5.2 POST MENOPAUSAL ENDOMETRIUM

Endometrial cancer is most commonly diagnosed in women after their menopause, so as well as using normal fertile samples, the use of post-menopausal endometrial samples as a control is the most useful.

The role and function of the four MIPs in pre-menopausal endometrium is still largely unknown. The functional involvement of the MIPs that may occur in the normal fertile endometrium may not be relevant in the endometria of post-menopausal women for obvious reasons. However, postmenopausal endometrium although not cycling due to lack of ovarian hormone cyclicity, still retains the ability to respond to ovarian hormones if available. Therefore although normally not functional, the functional potential is retained.

The immuno-staining of the MIPs on both the control groups, normal fertile women during the proliferative phase and postmenopausal endometrium, were analysed and compared to ascertain the most suitable control group to understand the functional relevance and also the diagnostic, clinical relevance of MIPs in endometrial cancers.

If the MIPs were absent in postmenopausal endometrium it could be significant marker of pre-malignant or malignant change. However, to confirm this hypothesis

larger number of normal postmenopausal endometrial samples; endometrial hyperplasia samples and early stage endometrial cancers will need to be studied in the future. One difficulty that can be foreseen is the expression of the MIPs in pre-menopausal endometrium.

One would envisage similar expression in the women on hormone replacement therapy and therefore MIPs may not be able to distinguish malignant changes if the women have been exposed to HRT. Since some HRT preparations increase the risk of development of endometrial cancer this may be an important question that need to be addressed in future studies

All postmenopausal samples showed a decreased immuno-reactivity of all the MIPs in comparison to normal fertile samples. When analysing immuno-staining of the MIPs in postmenopausal endometrium, Quick Score method was not used, due the limited amount of endometrium collected due to the extremely thin endometrium that is seen in healthy normal women without any endometrial pathology. Analysis was undertaken by assessing the intensity of staining along with descriptive localisation. This might have slightly skewed the results obtained when comparing both control groups. As results gained were all statistically significant we can still assume the results are valid and reliable therefore use it as foundation to plan future work.

It was difficult to extract RNA from postmenopausal endometrial biopsies due to the naturally thin endometrium in this phase resulting in RNA of insufficient quality and quantity, therefore the expression of MIPs was not investigated with using RT-PCR. This is an important downfall to consider and rectify when planning future work.

5.3 ENDOMETRIAL CANCER

All four MIPs have been shown to be expressed in breast cancer cells, and since similar to breast cancer, endometrial cancer is also an oestrogen dependent cancer it was hypothesised that the MIPs would be similarly expressed in endometrial cancer cells.

Results gained from this study agree with the above hypothesis for the MIPs S100A4 and S100P but not with AGR2 and OPN. Both IHC and RT-PCR have shown that endometrial cancer cells highly express S100A4 and S100P, but express less AGR2 in comparison to both normal fertile and postmenopausal endometrium. Furthermore, the results obtained from the two methods I used (IHC and RT-PCR) were contradictory for OPN, with IHC displaying a reduction in OPN expression in endometrial cancer cell, and RT-PCR demonstrating increased expression.

The MIPs may be expressed as a protein, but not necessary in mRNA, as it is less stable. The sample size for RT-PCR was too small in comparison with samples analysed by IHC (i.e. Two endometrial cancer samples were analysed for RT-PCR compared to 30 for IHC). With the RT-PCR we analysed the whole tissue sample including all cells (normal adjacent endometrial epithelial, stromal, blood vessel, blood cells and cancer cells) but with IHC only analysed an exact single cellular compartment of interest. This may be the main reason for the differences seen. The exact cellular composition of the frozen tissue biopsy that was used to extract RNA is not known so the resulting MIPs expression seen will only be a relative suggestion but may not be an exact match to the IHC results.

Since the IHC was done on paraffin tissue sections, the secondary confirmatory method of RT-PCR would be ideally done on the same biopsy. Some say the high level of paraffin in the section may alter the amplification of the DNA. This was demonstrated during optimisation of the RT-PCR technique, where S100A4 and OPN produced no positive results, despite adding amplification enhancing reagents. AGR2 and S100P, however, amplified the cDNA adequately using the RNA extracted from paraffin section. This may suggest that AGR2 and S100P are more sensitive in being locating and amplified in mRNA compared to S100A4 and OPN. RT-PCR is not quantitative analysis of the mRNA message; quantitative real time PCR may be a more suitable method for this.

No significant relationship was determined between endometrial cancer FIGO grade and expression of the MIPs. A larger sample set may be required in order for confirmatory statement on MIPs and FIGO grade correlation to be established.

5.3.a OSTEOPONTIN

Functionally, OPN mediates cell adhesion, chemotaxis, stress-dependent angiogenesis, prevention of apoptosis and anchorage-independent growth of tumour cells (Wai & Kuo 2004).

Conflicting results were generated with the two laboratory methods in assessing OPN expression. With the large difference in sample size used between the two methods, it is difficult to compare them directly. IHC showed a statistically significant decreased immuno-reactivity of OPN in endometrial cancer cells compared to normal fertile proliferative phase group (Mann Whitney U test; Glandular $p=0.0047$, Luminal $p =$

0.0018). However compared to postmenopausal control samples, immuno-reactivity of OPN was increased (Mann Whitney U test; Glandular p=0.0001, Luminal p < 0.0001).

In comparison to postmenopausal endometrium, results suggest that OPN may have properties, which aid in endometrial cancer metastasis. A wider and larger range of samples, in particular postmenopausal samples, needs to be examined to assess the difference in OPN expression.

5.3.b S100P

S100P has been shown to mediate tumour growth, drug resistance, and metastasis. Therefore, S100P is a useful marker for differentiating cancer cells from normal cells, which can aid in the diagnosis of cancer by cytological examination. There is considerable evidence demonstrating the elevated expression of S100P and tumour cell migration in various cancers (Sato & Hitomi 2002; Guerreiro Da Silva et al 2000; Beer et al 2002; Torres Schor et al 2006).

Results showed a statistically significant increased immuno-reactivity and expression of S100P in endometrial cancer cells compared to the control groups (Mann Whitney U test; N.Fertile Stroma p=0.0007), P.Meno Stroma p >0.0001), therefore concurring with previous reports made.

5.3.c AGR2

AGR2 has been widely investigated in breast cancer, showing an over expression of AGR2 in the cancer cells (Brychtova et al 2011; Liu et al 2005). It has also been show to be co-expressed with oestrogen receptor alpha (ER) in breast cancer cell lines and clinical specimens (Fletcher et al 2003)

The substantial evidence demonstrating the link between AGR2 and cancer cell migration, invasion and tumour growth lead us to hypothesise that endometrial cancer cells would aberrantly express AGR2.

Results from this study showed a decrease in AGR2 expression in endometrial cancer cells compared to normal fertile control group in both IHC and RT-PCR. However, the results were not statistically significant (Mann Whitney U test; Glandular $p=0.1736$, Luminal $p = 0.1175$). In comparison to postmenopausal endometrium there was a statistically significant increased immuno-reactivity to AGR2 in endometrial cancer cells (Mann Whitney U test; $p < 0.0001$).

RT-PCR results also confirmed the IHC results, but the difference observed was not statistically significant. This is primarily due to the small sample set used to assess the expression of AGR2 mRNA.

5.3.d S100A4

Studies to determine the function of S100A4 have shown that it may play a role in the different aspects of tumour progression, including motility, invasion, and apoptosis (Garrett et al 2006; Helfman et al 2005; Boye & Maelandsmo 2010).

Both IHC (MWU Test; NFertile Stromal p=0.0275, P.Meno Stromal p=0.0004) and RT-PCR demonstrated S100A4 to be over expressed in endometrial cancer cells, compared to both control groups. This suggests that up-regulation of S100A4 may have a direct role in inducing metastasis in endometrial cancer.

5.4 LIMITATIONS

As with all research there are limitations within the work carried out for this project. A greater sample size, particularly with RT-PCR would have enhanced the data generated, increasing the reliability and validity of results. Nevertheless majority of the findings have been well demonstrated and statistically significant, despite the sample size.

Quick Score technique was used to semi-quantitatively analyse and compare immunohistochemistry attaining for the four MIPs. Taking in consideration of size and integrity of each sample, this technique was only applied to analysing the normal fertile control and endometrial cancer samples. Due to the sparse amount of intact postmenopausal endometrium collected, the samples were subjectively analysed using generalised baseline intensity score and descriptive localisation remarks. This would undoubtedly lead to some discrepancies with the statistically analysis comparing the separate groups, however a generalised comparison was elicited. The analysis technique used in future studies would need to be further evaluated to ensure the accuracy of results.

Pathology reports determined two endometrial cancers, collected at Liverpool Women's Hospital, as carcinosarcoma. There is controversy over whether you can class these as true endometrial cancer. As discussed in Chapter 1.2, studies have included this as Type 2 endometrial cancers, but it does raise the point of the whether it can be a true comparison to Type 1 and Type 2 endometrial cancers. Likewise for the mixed endometrial carcinomas, one is not entirely sure which areas they are analysing, serous or endometrioid. Can the results be used to compare with a true endometrioid endometrial cancer? Ideally with a larger and wider range of samples, the groups could be split and individually compared to get an accurate and more reliable comparison.

Tissue samples used for the RT-PCR would need to be evaluated and considered when analysing results. The endometrial biopsy collected undoubtedly contains amounts of blood, mucus and myometrium from full thickness samples, and from the naked eye it is hard to distinguish what part of the biopsy has been included in the analysis.

5.5 FURTHER WORK

Further work is necessary to obtain more detailed an accurate results. Laser capture micro-dissection of specific endometrial cancer cells can be undertaken to ensure that RNA is only extracted from cells of interest, for example dissected endometrial cancer cells.

Obtaining samples of endometrial metastatic lesions would be ideal in assessing the expression of the MIPs while comparing it to endometrial cancer cells in the endometrium. When analysing the demographic and patient data, it was clear that majority of the patients were diagnosed with early stage cancer (50% FIGO Grade 1 for endometrioid endometrial cancer and 76% FIGO Stage I all endometrial cancer). The number of women seen with metastatic endometrial cancer is low, additional surgery is not the primary treatment for high grade/stage cancers. Therefore obtaining samples of endometrial metastatic lesions is a challenge, practically and ethically.

Further laboratory techniques, including quantitative real time RT-PCR and Western blotting should be done to confirm the results gathered from immunohistochemistry. Long-term follow up of endometrial cancer patients would also allow for a detailed assessment of progress of the cancer.

Functional studies of in-vitro primary endometrial cancer cells or endometrial cancer cell lines need to be undertaken for reproducibility. We have successfully cultured and maintained the growth of an endometrial cancer sample in our laboratory, producing a primary endometrial cancer cell line. See Fig 44.

Using these cells we can further investigate the role of the four MIPs and investigate the function of MIPs in live cells in culture (in vitro). For example we can inhibit MIPs expression (by silencing MIPs gene expression using techniques such as short interfering RNA (siRNA)) of cultured primary endometrial cancers and then assess the changes in the invasive / migratory potential of those cells in vitro to confirm the function of MIPs in the metastatic process. Furthermore established cell lines can be also be utilised to investigate the MIPs further in established invasion, adhesion assay systems and also by xenografting such cell lines in immune-compromised mice.

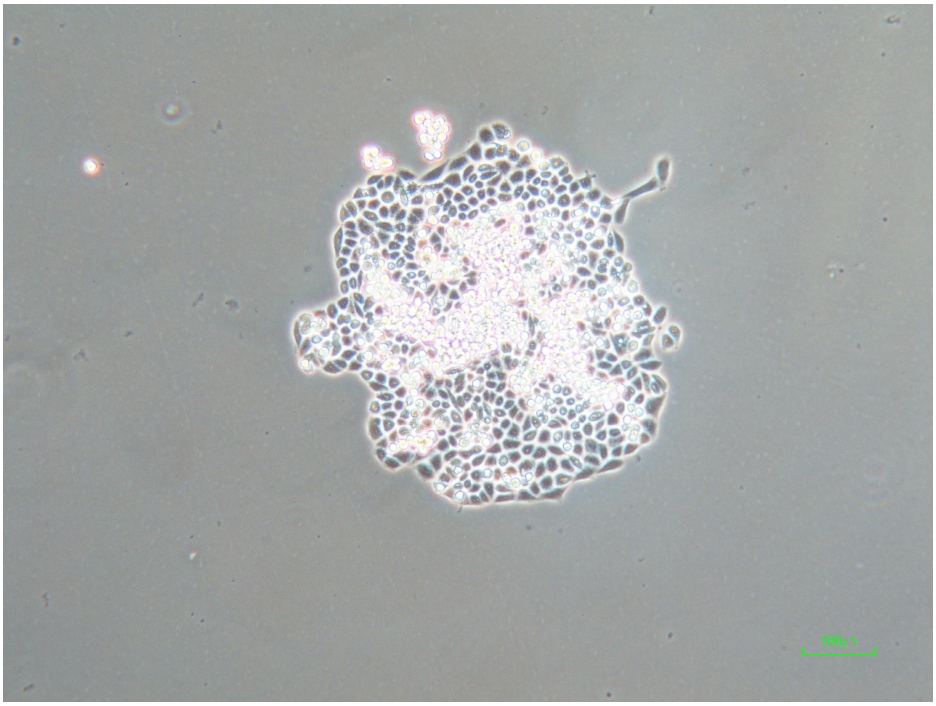


Figure 44: Phase micrograph of endometrial cancer (Courtesy of AJ Valentijin)

At the moment we cannot say whether MIP expression is a causative (essential) or resultant effect of endometrial cancer. This question however, can only be answered in future in vitro studies using direct transfection of expression vectors for the MIPs into endometrial caner cell lines and testing for the ability of the resultant transfectants to produce endometrial cancer lesions in syngenic animal models.

It has been shown that endometrial cancer cells have a small, specialised population of cancer stem/progenitor cells (CSCs) and these are speculated to give rise to distant metastasis (Hubbard et al 2009). We are therefore currently assessing the expression of cell fate markers CD133, CD138 and Telomerase in endometrial cancer. These cell fate markers regulate the survival ability of the endometrial cells, contributing to endometrial cancer tumorigenicity, pathology, hence the metastatic process (Wu & Wu 2009; Rutella et al 2009; Kodama et al 2005; Kyo et al 1997; Brien et al 1997).

5.6 SUMMARY

Overall the data from this project provides an interesting insight into the metastatic process of endometrial cancer. Our results suggest that all four MIPs play a key role in metastasis of endometrial cancer. Further research is required to understand the extent of the involvement OPN, S100P, AGR2 and S100A4 in inducing the metastatic process.

As one of the most common gynaecological malignancies, and the increasing incidence and mortality rates, it is important to create a non-invasive and reliable assay of detecting endometrial cancer early. With further advanced research these four MIPs could play a pivotal role in providing very useful diagnostic markers for endometrial cancer.

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7. APPENDIX

7.1 ENDOMETRIAL STEM CELL STUDY ETHICS

National Research Ethics Service
North West 2 Research Ethics Committee - Liverpool Central
Room 181
Gateway House
Piccadilly South
Manchester
M60 7LP

Telephone: 0161 237 2336
Facsimile: 0161 237 2383

05 October 2009

Dr Dharani K Hapangama
Clinical Senior Lecturer / Honorory Consultant in Obstetrics & Gynaecology
University of Liverpool
University Department, First Floor
Liverpool Women's Hospital,Crown St
Liverpool
L8 7SS

Dear Dr Hapangama

Study Title: **The role of the identified regulators of cell fate (RCF) and metastasis-inducing-proteins (MIP) in endometrial stem/progenitor cells (SPC) in endometriosis**
REC reference number: **09/H1005/55**
Protocol number: **1.0**

Thank you for your letter of 14 September 2009, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered in correspondence by a sub-committee of the REC. A list of the sub-committee members is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research

governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk. *Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.*

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		
REC application	2.2	
Protocol	1.0	02 July 2009
Investigator CV		
GP/Consultant Information Sheets		
Letter from Sponsor		02 July 2009
Referees or other scientific critique report		
Participant Information Sheet	2	09 September 2009
Participant Consent Form	2	09 September 2009
poster healthy + baby		
poster endometriosis		
Response to Request for Further Information		14 September 2009

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of

changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H1005/55	**Please quote this number on all correspondence**

Yours sincerely

Professor Sobhan Vinjamuri
Chair

Email: carol.ebenezer@northwest.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*

"After ethical review – guidance for researchers"

Copy to: *Mrs Gillian Vernon*

North West 2 Research Ethics Committee - Liverpool Central

Attendance at Sub-Committee of the REC meeting on 05 October 2009

Committee Members:

Name	*Profession*	*Present*	*Notes*
Mrs Sheila Gill	Lay Member	Yes	
Mr G Gilling-Smith	Consultant Vascular Surgeon	Yes	
Professor Sobhan Vinjamuri	Consultant in Nuclear Medicine	Yes	

Liverpool Women's
NHS Foundation Trust

Ethics Submission No: 09/H1005/55

PATIENT INFORMATION SHEET

"Endometrial stem cell Study"

The role of the identified regulators of cell fate (RCF) and metastasis-inducing-proteins (MIP) in endometrial stem/progenitor cells (SPC) in endometriosis.

Version 1: Endometrial biopsy only

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this

Why are we doing the study?

The inner lining of the womb (endometrium) may play an important part in endometriosis. **Endometriosis** is a common condition in which patches of the inner lining of the womb appear in parts of the body other than the cavity of the womb and seen in 1 in 10 women below the age of 50. It can cause painful periods, pelvic pain, pain with sexual intercourse and infertility. It is possible that an abnormality of specials cells in the endometrium called stem cells can cause endometriosis. If so, the information from this study will help us to develop new techniques to diagnose and treat this distressing condition.

What are stem cells?

Stem cells are special cells that can renew themselves (adult stem cells), and their job in the body is not yet determined. The inner-lining of the womb (endometrium) has these stem cells that can become many different types of cells, and they are likely to be responsible for its monthly regeneration. With monthly bleeding these cells are shed and can be expelled into the abdominal cavity. If these cells are implanted in the pelvis they can cause endometriosis as endometriosis occurs when endometrial cells of are found growing outside of the womb. We believe that abnormalities of these stem cells may cause endometriosis.

Why have I been chosen?

We are looking for a total of 160 women (you must have been off all hormonal medicines for at least 3 months), who have regular periods. We are specifically looking for 80 women who have endometriosis and another 80 completely healthy women who have had at least one baby. If you belong to any of these groups we will ask you if you would want to take part in the study.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

1. *If you are having a hysterectomy:*
   Your operation will continue exactly as planned. However, once the operation is over, a small sample of the womb that has already been removed will be taken for the research.
2. *If you are not having a hysterectomy:*
   Your operation will continue exactly as planned. However, a pipelle sample (see below) will also be taken from you for research.

The sample of endometrium will be processed in the lab to isolate the stem cells from it. Therefore, **NO** extra surgery will be performed for the study. A **blood sample** (5mls = teaspoonful of) will also be taken from your veins.

How is the endometrial pipelle sample done?

Whilst you are in the clinic (without anaesthetic) or whilst you are under anesthetic, the doctor will place a speculum (just like when you have a cervical smear) in the vagina. A plastic instrument (like a blunt drinking straw) will then be introduced through the neck of your womb to gently suck some cells from the inner lining of the womb. These cells will be then sent to the laboratory to be examined. This procedure is routinely done in our Gynaecology clinic and apart from the mild lower abdominal period like discomfort and vaginal spotting, it does not usually cause other problems. If you are going to have the biopsy taken at the time of the operation under anesthetic, you will not have any extra discomfort.

What are the possible benefits of taking part?

We do not expect you to have any extra benefits by taking part in this study. However, you will be helping us to improve our knowledge about endometriosis.

What if something goes wrong?

There are no special compensation arrangements in place to compensate you in case if taking part in this research project harms you. However, if you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Data management

The information gathered from this study will be entered into a computer database and will be analyzed in a strictly confidential manner, in compliance with the Data Protection Act. Regulatory authorities for approving medicines and the University of Liverpool may wish to look at medical records to check that the study has been performed correctly. All information, which is collected about you during the course of the research will be kept

strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognized from it. Once we carryout the study on the samples you kindly donate, if there is any surplus tissue, it will be stored in the department of obstetrics & Gyanecology and will be used in other ethically approved studies.

If you are interested in taking part, please contact Dr. Dharani Hapangama (0151- 702 4114 or 0151 706 9988) in the Liverpool Women's Hospital, Crown Street, Liverpool.

If you want to find out more about the study from someone who is not directly involved in it and can give you unbiased advice, please contact Mr Jonathan Herod, Consultant, in Gynaecology Out Patient Clinic, telephone no. 0151 708 9988.

Liverpool Women's
NHS Foundation Trust

Ethics Submission No: 09/H1005/55
PATIENT INFORMATION SHEET

"Endometrial stem cell Study"

Role of endometrial stem cells in endometriosis.

Version 2: Biopsy of Endometrium and endometriosis lesions

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this

Why are we doing the study?

The inner lining of the womb (endometrium) may play an important part in endometriosis. **Endometriosis** is a common condition in which patches of the inner lining of the womb appear in parts of the body other than the cavity of the womb and seen in 1 in 10 women below the age of 50. It can cause painful periods, pelvic pain, pain with sexual intercourse and infertility. It is possible that an abnormality of specials cells in the endometrium called stem cells can cause endometriosis. If so, the information from this study will help us to develop new techniques to diagnose and treat this distressing condition.

What are stem cells?

Stem cells are special cells that can renew themselves (adult stem cells). The inner-lining of the womb (endometrium) has these stem cells that are likely to be responsible for its monthly regeneration. With monthly bleeding these cells are shed and can be expelled into the abdominal cavity. If these cells are implanted in the pelvis they can cause endometriosis as endometriosis occurs when endometrial cells of are found growing outside of the womb. We believe that abnormalities of these stem cells may cause endometriosis.

Why have I been chosen?

We are looking for a total of 80 women with endometriosis (you must have been off all hormonal contraception for at least 3 months), who have regular periods. If you have endometriosis and is scheduled to have surgery to remove endometriosis lesions we will ask you if you would want to take part in the study.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

1. *If you are having a hysterectomy:*

Your operation will continue exactly as planned. However, once the operation is over, a small sample of the womb that has already been removed will be taken for the research.

2. *If you are not having a hysterectomy:*

Your operation will continue exactly as planned. However, a pipelle sample (see below) will be taken from you. Once the operation is over, a small sample of the endometriosis tissue that has already been removed will also be taken for the research.

Therefore, **NO** extra surgery will be performed for the study. A ***blood sample*** will also be taken from your veins.

How is the endometrial pipelle sample done?

Whilst you are under anesthetic, the doctor will place a speculum (just like when you have a cervical smear) in the vagina. A plastic instrument (like a blunt drinking straw) will then be introduced through the neck of your womb to gently suck some cells from the inner lining of the womb. These cells will be then sent to the laboratory to be examined. This procedure is routinely done in our Gynaecology clinic and apart from the mild lower abdominal period like discomfort and vaginal spotting, it does not usually cause other problems. Since you are going to have the biopsy taken at the time of the operation under anesthetic, you will not have any extra discomfort.

What are the possible benefits of taking part?

We do not expect you to have any extra benefits by taking part in this study. However, you will be helping us to improve our knowledge about endometriosis.

What if something goes wrong?

There are no special compensation arrangements in place to compensate you in case if taking part in this research project harms you. However, if you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Data management

The information gathered from this study will be entered into a computer database and will be analyzed in a strictly confidential manner, in compliance with the Data Protection Act. Regulatory authorities for approving medicines and the University of Liverpool may wish to look at medical records to check that the study has been performed correctly. All information, which is collected about you during the course of the research will be kept strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognized from it. Any surplus tissue after being used for this study, will be stored in the Liverpool Women's Hospital and may be used for other ethically approved research.

If you are interested in taking part, please contact Dr. Dharani Hapangama (0151- 702 4114 or 0151 706 9988, bleep 141) in the Liverpool Women's Hospital, Crown Street, Liverpool.

If you want to find out more about the study from someone who is not directly involved in it and can give you unbiased advice, please contact Mr Jonathan Herod, Consultant, in Gynaecology Out Patient Clinic, telephone no. 0151 708 9988.

Liverpool
Women's
Hospital

Study Number:
Patient Identification Number for this trial:

CONSENT FORM

Title of Project: **The role of the identified regulators of cell fate (RCF) and metastasis-inducing-proteins (MIP) in endometrial stem/progenitor cells (SPC) in endometriosis.**

Name of Researcher: Dr Dharani Hapangama, Senior Lecturer
University of Liverpool / Liverpool Women's Hospital

Please initial box

1. I confirm that I have read and understand the information sheet dated ..... (version .....) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from [University of Liverpool & Liverpool Women's Hospital] or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree to take part in the above study and for my GP to be informed of my part taking. ☐
5. I agree for surplus tissue to be stored in the department of obstetrics & Gyanecology and to be used in other ethically approved studies. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

SDS0210M P.I.S. (February 2005)

Liverpool Women's
NHS Foundation Trust

Study number: LWH0813
Patient Identification Number for this trial:

Title of Project: The role of the identified regulators of cell fate (RCF) and metastasis-inducing-proteins (MIP) in endometrial stem/progenitor cells (SPC) in endometriosis.

Researcher: Dr Dharani Hapangama
Clinical Senior Lecturer / Consultant in Obstetrics & Gynaecology Division of Perinatal and Reproductive Medicine School of Reproductive and Developmental Medicine First Floor Liverpool Women's Hospital Crown Street Liverpool L8 7SS

Age:

Height (cm):
Weight (kg):
Body mass index:

Smoking history:

Parity:
Miscarriage:
TOP:

Days of bleeding:
Cycle length:
LMP:

Endometriosis stage:

7.2 MIPS IN ENDOMETRIAL CANCER

Liverpool Women's NHS
NHS Foundation Trust

Crown Street
Liverpool
L8 7SS

Tel: 0151 708 9988
www.lwh.nhs.uk

14th April 2011

Dr Dharani Hapangama
Clinical Senior Lecturer / Honorary Consultant in O&G
Liverpool Women's Hospital
University Department, First Floor
Crown Street
Liverpool L8 7SS

Direct dial: 0151 702 4346
Email: Gillian.vernon@lwh.nhs.uk

Dear Dharani

ID: LWH0877 – Study of the Role of Metastasis Inducing Proteins and cell fate regulators in the pathogenesis of Endometrial Cancer

Following submission of project documents, associated paperwork and approvals to the Trust's R&D Department, I am pleased to inform you that your research project has been approved by the R&D Director. This approval relates to the documentation listed below:

- Ethics approval letter [11/H1005/4] dated 11th April 2011
- Protocol [version 1.0] 9th December 2010

The research is registered on the Trust's R&D database under the reference LWH0877, which I would be grateful if you could quote in all future correspondence regarding the project.

The Sponsor(s) of this research project under the Research Governance Framework for Health and Social Care (RGF) are the Trust and the University of Liverpool.

Having gained approval to conduct this research under the auspices of Liverpool Women's NHS Foundation Trust, you will be expected to comply with the principles and guidelines set out in ICH Good Clinical Practice and the Department of Health RGF. Please refer to your delegated duties outlined overleaf.

I would like to take this opportunity to wish you the best of luck with this research and to request a copy of the final report and any subsequent publications.

Yours sincerely

Gillian Vernon
Research & Development Manager

Liverpool Women's

UNIVERSITY OF LIVERPOOL

Liverpool Women's NHS
NHS Foundation Trust

Ethics Submission No: 11/H1005/4

PATIENT INFORMATION SHEET

"MIPs in Endometrial Cancer Study"

Role of metastasis-inducing-proteins in endometrial cancer

Version 1.1: For patients undergoing hysterectomy.

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this

Why are we doing the study?

Each year in the UK about 4500 women, commonly in their 50s and 60s, develop cancer of the lining of the womb (endometrium). The 'endometrium' builds up and is then shed each month as a 'period' before menopause. In women who unfortunately develop cancer, the cells in the endometrium multiply and behave abnormally. The survival rate of early stage endometrial cancer is good, however in extreme cases cancer cells can spread beyond the womb affecting the overall outcome of the disease. We are doing this study to better understand the changes that happen in endometrial cells. This will help us discover new targets to diagnose and design new treatment for endometrial cancer.

What is metastasis?

The spread of cancer cells beyond their origin, the womb, is called 'metastases'. This metastatic process is closely linked with the outcome of the disease, but is not fully understood. It is possible that there are special proteins that can encourage cancer cells to spread. These specific proteins are called 'metastasis-inducing-proteins' (MIPs), which we believe can cause cancer cells to invade healthy tissue. We would like to investigate the presence of these proteins in endometrial cancer cells and the role they play.

Why have I been chosen?

We are looking for a total of 160 women who are undergoing hysterectomy. We are specifically looking for 80 women who have endometrial cancer and another 80 healthy women undergoing surgery. If you belong to any of these groups we will ask you if you would want to take part in the study.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You are free to withdraw at any time and without giving a reason. The decision to not take part or withdraw at any time, will **NOT** affect the standard of care you receive.

What will happen to me if I take part?
Your operation will continue exactly as planned. However, once the operation is over, a small sample of the womb that has already been removed from you will be taken for the research.
Therefore, **NO** extra surgery or procedures will be performed for the study. A **blood sample** will also be taken from your veins.

What are the possible benefits of taking part?
We do not expect you to have any extra benefits by taking part in this study. However, you will be helping us to improve our knowledge about endometrial cancer.

What if something goes wrong?
We do not anticipate any harm to arise while taking part in this study, as we are not carrying out any additional procedures. However, if you are harmed due to someone's negligence, then you may have grounds for a legal action. There are no special compensation arrangements in place to compensate you in case if taking part in this research project harms you. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Data management
The information gathered from this study will be entered into a computer database and will be analyzed in a strictly confidential manner, in compliance with the Data Protection Act. Regulatory authorities for approving medicines and the University of Liverpool may wish to look at medical records to check that the study has been performed correctly. All information, which is collected about you during the course of the research, will be kept strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognized from it.

Surplus tissue
You can chose to give consent for any remaining tissue, after being used for this study, to be anonymised and stored in the Liverpool Women's Hospital which can be used for future ethically approved research.

If you are interested in taking part, please contact Dr. Dharani Hapangama (0151- 702 4114 or 0151 706 9988, bleep 141) in the Liverpool Women's Hospital, Crown Street, Liverpool.

If you want to find out more about the study from someone who is not directly involved in it and can give you unbiased advice, please contact **Mr Jonathan Herod, Consultant Gynaecology Oncologist, in Gynaecology Out Patient Clinic, telephone no. 0151 708 9988.**

Hapangama / Endo Version 1.1 **Date: 6th April 2011**

UNIVERSITY OF LIVERPOOL

Liverpool Women's NHS
NHS Foundation Trust

Ethics Submission No: 11/H1005/4

PATIENT INFORMATION SHEET

"MIPs in Endometrial Cancer Study"

Role of metastasis-inducing-proteins in endometrial cancer

Version 2.1: For patients undergoing Pipelle Sampling

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this

Why are we doing the study?

Each year in the UK about 4500 women, commonly in their 50s and 60s, develop cancer of the lining of the womb (endometrium). The 'endometrium' builds up and is then shed each month as a 'period' before menopause. In women who unfortunately develop cancer, the cells in the endometrium multiply and behave abnormally. The survival rate of early stage endometrial cancer is good, however in extreme cases cancer cells can spread beyond the womb affecting the overall outcome of the disease. We are doing this study to better understand the changes that happen in endometrial cells. This will help us discover new targets to diagnose and design new treatment for endometrial cancer.

What is metastasis?

The spread of cancer cells beyond their origin, the womb, is called 'metastases'. This metastatic process is closely linked with the outcome of the disease, but is not fully understood. It is possible that there are special proteins that can encourage cancer cells to spread. These specific proteins are called 'metastasis-inducing-proteins' (MIPs), which we believe can cause cancer cells to invade healthy tissue. We would like to investigate the presence of these proteins in endometrial cancer cells and the role they play.

Why have I been chosen?

We are looking for a total of 160 women. We are specifically looking for 80 women who have endometrial cancer and another 80 healthy women undergoing surgery. If you belong to any of these groups we will ask you if you would want to take part in the study.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You are free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?
Your operation will continue exactly as planned. However, a pipelle sample (see below) will also be taken from you for research.
Therefore, **NO** extra surgery will be performed for the study. A **blood sample** will also be taken from your veins if you are still having monthly periods.

How is the endometrial pipelle sample done?
Whilst you are in the clinic (without anaesthetic) or whilst you are under anesthetic, the doctor will place a speculum (just like when you have a cervical smear) in the vagina. A plastic instrument (like a blunt drinking straw) will then be introduced through the neck of your womb to gently suck some cells from the inner lining of the womb. These cells will be then sent to the laboratory to be examined. This procedure is routinely done in our Gynaecology clinic and apart from the mild lower abdominal period like discomfort and vaginal spotting, it does not usually cause other problems. If you are going to have the biopsy taken at the time of the operation under anesthetic, you will not have any extra discomfort.

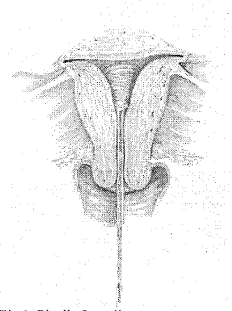


Fig 1: Pipelle Sampling

What are the possible benefits of taking part?
We do not expect you to have any extra benefits by taking part in this study. However, you will be helping us to improve our knowledge about endometrial cancer.

What if something goes wrong?
We do not anticipate any harm to arise while taking part in this study, as we are not carrying out any additional procedures. However, if you are harmed due to someone's negligence, then you may have grounds for a legal action. There are no special compensation arrangements in place to compensate you in case if taking part in this research project harms you. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Data management
The information gathered from this study will be entered into a computer database and will be analyzed in a strictly confidential manner, in compliance with the Data Protection Act. Regulatory authorities for approving medicines and the University of Liverpool may wish to look at medical records to check that the study has been performed correctly. All information, which is collected about you during the course of the research will be kept

strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognized from it.

Surplus tissue
You can chose to give consent for any remaining tissue, after being used for this study, to be anonymised and stored in the Liverpool Women's Hospital which can be used for future ethically approved research.

If you are interested in taking part, please contact Dr. Dharani Hapangama (0151- 702 4114 or 0151 706 9988, bleep 141) in the Liverpool Women's Hospital, Crown Street, Liverpool.

If you want to find out more about the study from someone who is not directly involved in it and can give you unbiased advice, please contact Mr Jonathan Herod, Consultant, in Gynaecology Out Patient Clinic, telephone no. 0151 708 9988.

Liverpool Women's NHS
NHS Foundation Trust

Patient Identification Number for this trial:

CONSENT FORM

Title of Project: The role of metastasis-inducing-proteins (MIP) in endometrial cancer

Name of Researcher: Dr Dharani Hapangama, Senior Lecturer
University of Liverpool / Liverpool Women's Hospital

Please initial box

1. I confirm that I have read and understand the information sheet dated ..... (version .....) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from [University of Liverpool & Liverpool Women's Hospital] or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree for surplus tissue to be stored in the department of obstetrics & Gynaecology and to be used in other ethically approved studies. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

7.3 STANDARD OPERATING PROTOCOLS

7.3.a SAMPLE RECEPTION: PROCESSING ENDOMETRIAL BIOPSIES

BACKGROUND

The SOP will describe the protocol for the fixation of endometrial biopsy samples in 10% neutral buffered formalin and preparation of frozen blocks in liquid nitrogen. The SOP will also briefly describe the archive arrangements for the sample and patient information.

HEALTH AND SAFETY REQUIREMENTS

Laboratory workers should be immunised against hepatitis B before commencing work with human material (refer to the University of Liverpool code of practice on immunisation available from the departmental safety coordinators office). Laboratory coats and nitrile gloves should be worn at all times when handling the unfixed biopsies. All manipulations on the fresh samples should be performed in the class II microbiological safety cabinet situated in room 1124.

Care should be taken when dealing with dry ice and liquid nitrogen due to risks of burns and asphyxiation. The researcher should work in a well-ventilated room and wear personal protection equipment including thermal gloves and full safety face shield. Refer to the endometrial sample reception risk assessment and the specific COSHH forms for further information. Guidelines on the use of cryogenic substances are also available from the physical hazards code of practice on the University of Liverpool health and safety intranet:
https://www.liv.ac.uk/intranet/safety/codes_of_practice/physical_hazards.pdf

In the event of a biological spillage onto the skin, sharps or burn injury, wash immediately with cold running tap water. Seek immediate advice from the designated first aider and report the incident to the Departmental Safety Coordinator within 24 hours.

EQUIPMENT INFORMATION:

- Dry ice, Virkon and laboratory plastics (e.g. universals etc) are purchased from Liverpool Women's Hospital (NHS) purchasing department.
- Nunc cryotubes are purchased from Corning. Distributor address: PO Box 75089, 1117 ZP Schiphol, Koolhavenlaan, The Netherlands.
- Forceps and scissors are supplied by Raymond A Lamb Ltd. Manufacturer's address: Units 4 & 5, Parkview industrial estate, Eastbourne, East Sussex, BN23 6QE England.
- TriGene Advance is obtained from Medichem International. Supplier address: PO Box 237, Seven Oaks, Kent, TN15 0ZJ. Telephone: 01732 763555.
- Neutral Buffered Formalin solution is purchased from Sigma Aldrich. Supplier address: Fancy road, Poole, Dorset, BH12 4QH. Telephone 01202 712300.
- Biomat^2 Recirculating microbiological safety cabinet was purchased from Bioquell. Supplier information: Walworth road, Andover, Hampshire, SP10 5AA.
- Cool Jet Instant Freezing Aerosol purchased from Raymond A Lamb. Supplier information: Units 4 and 5 Parkview Industrial Estate, Eastbourne, East Sussex, BN23 6QE England. Telephone 01323737000

METHOD:

A Laboratory Preparation:

1 Lift glass shield of microbiological safety class II (MCII) cabinet until edge is level with the second notch marked on the cabinet frame. Press and hold down the "ON" button until the MCII cabinet switches on.  ss and hold down to switch on light.

2 Clean the surfaces of the MCII cabinet with 1% TriGene Advance solution and dry with absorbent towels.

3 Place the following items on a clean plastic tray in the MCII cabinet:

- Universal tube containing phosphate buffered saline (PBS) and tissue. Tube should be labelled with the date and the specimen ID number.
- Small weighing boats
- Forceps and scissors
- Discard pot containing 1% Virkon solution.
- Equipment decontamination container (small plastic container filled to approximately 50% capacity with 2% TriGene Advance Solution).

4 A box of dry ice should be placed on the adjacent bench.

5 Label a Nunc cryotube with the specimen ID number and date.

6. Place the labelled cryotube and a clean pair of forceps in the box of dry ice to cool.

B Biopsy Processing Procedure:

In theatre endometrial tissue is obtained via pipelle sampling or full thickness endometrial biopsies. After biopsy tissue sample should be adequately distributed in the following tubes:

1. neutral based formalin (NBF)
2. PBS
3. culture media
4. RNA later solution

1 All tubes except for tube 1 should be placed immediately into crushed ice. Transport sample collection box to room 1124. Transport of human samples within the hospital should follow the University and Liverpool Women's Hospital Trust safety guidelines (refer to transport SOP for more information).

2 Store tube 1 under fume hood located in room 1125. Tissue in NBF tube should be left for 24 hours for next day processing. If the sample is collected on a Friday then the NBF pot should be stored in the sample reception fridge in room 1124 to minimise evaporation.

3 Tubes 3 and 4 should be placed in the refrigerator located in room 1124.

4 Transfer tube 2 to the MCII cabinet.

5 Place equipment decontamination container under hood.

6 Prepare box of dry ice and place in cabinet. Dry ice box should contain opened cryotube and forceps.

7 Transfer tissue from tube 2 to a weighing boat using a second pair of forceps. Carefully remove mucous and blood clots from the tissue and place waste in the 1% Virkon discard pot.

8 Using a non-sterile plastic pipette transfer 1% virkon to tube 2 containing remaining PBS for overnight decontamination

9 Manipulate the sample in the weighing boat so that it is coiled into a 'Danish pastry' shape with forceps. Discard used forceps in equipment decontamination container containing 2% trigene.

10 To freeze sample, place weighing boat on dry ice and using cool jet aerosol spray the top part of tissue sample until frozen. To loosen tissue sample in weighing boat rub bottom surface of weighing boat.

11 Transfer the tissue into the labelled cryotube using the pre-cooled forceps and replace lid. Discard second used forceps in equipment decontamination container when finished.

12 Place cryotube in the next available slot in the Nalgene storage boxes in freezer 3 (stack A, shelf 3 of the -80^0C Sanyo freezer situated in room 1127).

13 Enter the specimen ID number and date into the box plan in the freezer file (black ring binder held in room 1127).

14 Update the sample reception log (in room 1124). Enter sample ID, date and name of person processing samples. Enter information according to Good Clinical Laboratory Practice (GCLP) guidelines.

15 Complete both portions of the Biopsy request form, held in the RM Biopsy Log Ring binder. Enter patient ID number, specimen code, number of tissue pieces and sample storage location.

16 Detach top portion of the Biopsy request form and staple to the photocopied consent form. Forms are then placed into the locked filing cabinet in room 1127.

17 Original consent form is held by Dr Dharani Hapangama.

7.3.b SAMPLE RECEPTION: PROCESSING BLOOD SAMPLES

HEALTH AND SAFETY

Gloves should be worn whenever handling biological samples. Refer to COSHH and local guidelines before commencing with procedure.

BACKGROUND

Whole blood and serum collection is vital to gain information about oestrogen and progesterone hormone concentrations. Together with histological dating and the LMP this will allow an accurate detection of what time in the ovarian cycle the participant is in.

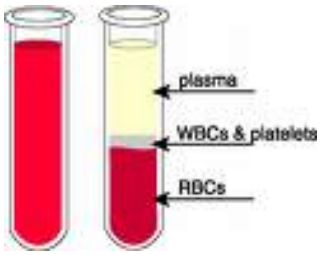
In is important to handle blood samples under the microbiological safety cabinet in lab 3 for protection against potentially hazardous micro-organisms.

EQUIPMENT INFORMATION

1. Dry ice, Virkon, blood bottles and laboratory plastics (e.g. pastettes) are purchased from Liverpool Women's Hospital (NHS) purchasing department.
2. Nunc cryotubes are purchased from Corning. Distributor address: PO Box 75089, 1117 ZP Schiphol, Koolhavenlaan, The Netherlands.
3. TriGene Advance is obtained from Medichem International. Supplier address: PO Box 237, Seven Oaks, Kent, TN15 0ZJ. Telephone: 01732 763555.
4. Biomat^2 Recirculating microbiological safety cabinet was purchased from Bioquell. Supplier information: Walworth road, Andover, Hampshire, SP10 5AA.
5. Hermle Z216MK Refrigerated Microcentrifuge purchased from Progen Scientific
   A division of G.H.Zeal Ltd. Zeal House 8, Deer Park Road, Merton, London, SW19 3UU

METHOD

1. Collect 2.7ml of blood in a pink EDTA tube, and 4.7mls of blood in a gold serum tube for plasma serum. 7.5mls of blood can be pre-collected in a 10ml syringe first and then distributed into the appropriate opened blood bottles. Replace the bottle cap and immediately invert both bottles 5 times.
2. Once obtained, immediately place both tubes in crushed ice until blood samples are transported to the labs, in room 1124.
3. Once transported into the lab, leave serum blood bottle to stand for at least 30 mins in refrigerator before centrifuging. Foam containers should be available to keep serum blood bottles in an upright position.
4. Unscrew lid to pink blood bottle. Using a blue -1000µl pipette, transfer whole blood from pink tube into two labelled cyrotubes (no centrifuging required). Replace cyrotube lids and place into dry ice box. Eject blue pipette tip into a container containing 2% virkon.
5. After 30 minutes, centrifuge serum blood tube at 3,000 rpm for 10 min at 4ºC. Please refer to Standard Operating Protocol 36 for "Use of the Hermle Z216MK Refrigerated Microcentrifuge".
6. Once centrifuged, carefully remove the sample bottle from the centrifuge machine in order to leave the layers undisturbed.



7. Place a clean blue tip on the pipette, remove the lid from the serum tube and carefully aspirate the serum plasma, distributing it to two labelled cyrotubes. Remove as much as possible, taking care not to disturb the buffy coat at the interface between the red cells and the plasma. Place cyrotubes into dry ice box.
8. Eject the blue pipette tip to a container containing virkon.
9. Cyrotubes should be labelled with:

- Specimen ID
- Contents, i.e. “whole blood” or “serum”
- Date obtained.

10. Carry dry ice box to room 1127 and place into the appropriate sample boxes in the -70°C freezer and record its exact location in the freezer file and fill blood sample forms.
11. Using a pastette transfer 2% virkon into both used blood bottle for disinfection purposes, and dispose blood bottles into clinical waste bin.
12. Clean fume hood after use using trigene spray.

7.3.c TISSUE PROCESSING

BACKGROUND

Formalin fixed tissue is dehydrated, cleared then impregnated with paraffin wax using the automated Shandon Citadel 1000 processing machine. The processing setting for most sample types is ***programme A*** which operates using the schedule provided below. The total processing time is 18 ¾ hours, therefore the processor is generally run overnight. Starting the processor at 2pm will result in completion of the cycle at 8:45 the following morning.

Programme A Processing Schedule:

4% formalin in neutral buffer	45 minutes
60% Ethanol	1 hour
70% Ethanol	1 hour
90% Ethanol	1 hour
100% Ethanol	1 hour

100% Ethanol	1 ½ hours
100% Ethanol	2 hours
Xylene 1	1 hour
Xylene 2	1 ½ hours
Xylene 3	2 hours
Wax 1	2 ½ hours
Wax 2	3 ½ hours

EQUIPMENT INFORMATION:

Citadel processor (serial number CA 1390 EO 608) manufacturer: Thermo electron Corporation. Manufacturer's address: 93 – 96 Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR England.

MAINTENANCE (CONTRACT AND SERVICING INFORMATION)

Maintained/serviced by Thermo Fisher Scientific 93 – 96 Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR England. Tel: 01928 562 541, Fax: 01928 562 512.

SOLVENT INFORMATION:

Ethanol and xylene are purchased from Chemistry solvent stores (University of Liverpool). The wax (Histoplast PE REF8330) is purchased from Thermo Scientific 93 – 96 Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR England. The 10% neutral buffered formalin is purchased from Sigma Life science, Fancy Road, Poole, Dorset, BH12 4QH.

HEALTH AND SAFETY PRECAUTIONS:

Wear a laboratory coat, safety spectacles and nitrile gloves. Xylene and formalin are harmful by contact to skin and eyes or if ingested or inhaled. Refer to the risk assessments and COSHH forms before starting procedure.

Overnight processing procedures requires the completion of permits provided in safety circular SCR14/3 (link to document provided below). A 'Yellow permit' (for long term operation) should be prepared by the DSC or laboratory manager and the copy secured in a clear plastic folder on the door outside laboratory 4. The 'Pink' permit (valid for the duration of the experiment only) should be prepared by the processor user and the document should be placed on or adjacent to the apparatus. Information that needs to be recorded on the pink copy includes:

a) experimental details

b) times and dates of experiment

c) emergency procedures

d) name, address and telephone number of the processor user

e) signature of researcher concerned and a countersignature from the laboratory manager/DSC.

PROTOCOL

A Preparation of Solvent Containers on Processing Machine:

Between processing runs, ethanol solutions are stored in labelled bottles to prevent evaporation. These are found in the metal solvent cabinet under the bench in Laboratory 4. Therefore the solvent containers must be re-filled before tissue processing commences. To access the processor solvent container, press the ***raise*** button on the hand held controller**.** Remove the evaporation covers from the top of the processor then press ***rotate*** or ***check/fill*** button. The rotate function will cause the operating head assembly to advance one position and the check/fill option rotates the head three positions. Remove the container using the metal handles and add 1.5L of the required ethanol solution (the top of the bevelled edge on the solvent container is approximately 1.5L). Repeat process until all ethanol containers are filled. It is also advisable to check the level of the xylene, formalin and the wax containers on a regular basis. If fresh 100% ethanol is used to top up or prepare solutions for the processor ensure the ethanol log is updated with the volume used. The ethanol log is situated in DSC office (room 1128).

B Sample Processing Procedure

1. Label plastic cassettes with the sample ID number using pencil.
2. Carefully remove the tissue from the formalin pot using forceps and place into the small wire inserts. Secure the inserts into the plastic cassettes and re-check the sample ID. Dispose of the used formalin in the red labelled waste container which is stored below the fume hood.
2. Arrange the cassettes so they are loosely spaced in the processing baskets
3. Press ***Go To 1*** on the hand held controller**,** slot the processing baskets onto the holder over the formalin and place weight on top of the basket.
4. Press ***Lower*** button on the hand held controller (to lower the basket into the formalin).
5. Check the time is correct by pressing ***clock*** (alter time by pressing the + button or whilst holding in the clock button if necessary).
6. Select the appropriate processing time function:

Date of Processing	**Processing Time**	**Hand Held Controller Action**
Monday-Thursday	Before 2pm	Press ***delay on*** button
Monday-Thursday	After 2pm	Press ***autostart*** button
Friday	Before 2pm	Press ***days delay,*** hold this in, scroll to 2 days using the + button then press ***delay on.***
Friday	After 2pm	press ***days delay,*** hold this in, scroll to 1 day using the + button, then press ***delay on***

7. Complete the citadel 1000 user log (the folder is situated by the embedding staition in laboratory 4) with sample, solvent and programme information..
8. When the processing cycles are completed, switch on the Shandon Histocentre 3 embedding machine.
9. Press ***raise*** button on the hand held controller and remove baskets from holder.
10. Place blocks into heated reservoir in the embedding machine.

11. Wipe processing baskets with absorbent towels to remove surplus wax then place equipment into the oven (heated to ~100°C) for several minutes to remove any residue.
12. Soak biopsy inserts in xylene for several hours/overnight to remove wax residues. Leave the inserts in the fume hood to air dry
13. Remove the alcohols from the processing machine using the method described in section A and pour solutions in the labelled storage bottles using a funnel. The storage bottles are then placed in the flammable solvent metal storage cabinets.
14. Update the citadel 1000 processing user log.

C Disposal of Waste Solvents

The solvents must be changed on a regular basis (depending on time of year and processor usage) to ensure efficient processing of the tissue samples. Waste solvents must be placed in the red solvent waste containers and identified clearly with the appropriate labels provided by Lisa Heathcote or Jo Drury. Contaminated waste wax can be discarded by pouring the molten solution into a container and cooling until the wax is set. The wax container can then be disposed of in the LWH clinical waste. Refer to the waste disposal protocol (SOP 25) for further information.

7.3.d EMBEDDING SAMPLES

BACKGROUND

The Shandon Histocentre provides easy convenient embedding of histological specimens. Prior to operating the unit, be sure to read and observe the safety instructions in the operator manual.

EQUIPMENT INFORMATION:

Plastic cassette

Shandon HistoCentre 3 Embedding Station

Hot forceps

Metal Moulds

HEALTH AND SAFETY REQUIREMENTS

Normal laboratory precautions, including the wearing of gloves, laboratory coat, safety goggles and the use of a fume cupboard, should be taken when handling the samples. Refer to the risk assessments and COSHH forms before starting procedure. Place all waste in clinical waste bin.

METHOD:

1. When the processing cycles are completed, switch on the Shandon Histocentre 3 embedding machine.
2. Press ***raise*** button on the hand held controller and remove baskets from holder (use paper towels to ensure that wax doesn't drip on the floor).
3. Place blocks into heated reservoir in the embedding machine.
4. Wipe processing baskets with absorbent towels to remove surplus wax then place equipment into the oven (heated to ~100°C) for several minutes to remove any residue.
5. Take a metal mould from the heated unit, place it underneath the wax tap and half-fill it with wax.
6. Take a cassette and place on the heated area by the wax tap to prevent solidification of the wax.
7. Open the cassette, and transfer the sample to the wax-filled mould using hot forceps.
8. Move the mould to the "cold spot" at the front of the dispensing unit
9. Position tissue to bottom surface of metal mould using hot forceps to stabilise tissue in place.
10. Cover with a plastic cassette (number upwards) and hold in place by applying pressure with hot forceps on top of cassette.
11. Top up wax by hovering above the heated area and place on pre-cooled cooling block.

12. Using forceps tap against top of plastic cassette to release any air bubbles within the liquid wax.
13. Leave on cooling area* for at least 30 minutes
14. Cassette should come cleanly away from the metal mould. If necessary use forceps or a scalpel to loosen the edges.
15. Store the block in the metal storage cabinet (trays 5 and 6) in numerical order (room 1125).
16. Place the metal biopsy inserts into a container of xylene to clean off the wax.

7.3.e CUTTING PARAFFIN SECTIONS

BACKGROUND

Preparation of 3-5 micron thick sections from wax – embedded tissue is a commonly used histological technique. This SOP will describe how to prepare these using the Microm HM335 rotary microtome and attaching sections onto aminopropyl triethoxy silane (APES) coated slides.

HEALTH AND SAFETY REQUIREMENTS

Wear a laboratory coat and nitrile gloves. Care needs to be taken when performing this procedure because there is a risk of sharps injury from the microtome blades. Refer to the physical hazards safety circular, risk assessments and COSHH forms before starting procedure. Also ensure waste wax sections are cleaned from the floor and the general working area at the end of the procedure to prevent risk of slip/trip injuries. Place all waste tissue sections in the clinical waste bags and the used microtome blades and slides into the yellow clinical waste sharps bins. DO NOT COMMENCE WORK WITH THE MICROTOME UNTIL SUFFICIENT TRAINING HAS BEEN PROVIDED AND AUTHORISATION HAS BEEN RECEIVED FROM LISA HEATHCOTE OR JO DRURY.

EQUIPMENT INFORMATION:

- Microm HM335 rotary microtome is supplied by MICROM Company. Manufacturer's address: Microm UK Ltd., 8 Thame Park Business Centre, Leinman Road, Thame, OX9 3XA. Further information can also be obtained on http://www.microm-online.com.
- Microscope slides (twin frost size 26 x 76mm-Printed "IVD CE" 90º ground edges, catalogue number MAE-1000-03P Pack of 1000) are purchased from Liverpool Women's Hospital (NHS) purchasing department.
- Coverslips 22 x 22mm, 22 x 40mm and 22 x 50 are purchased from Liverpool Women's Hospital (NHS) purchasing department.
- Microtome blades (MB Dynasharp Catalogue Reference 3050836) are purchased from Thermo Scientific 93 – 96 Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR England.
- Forceps, paint brush, section dryer and water bath are supplied by Raymond A Lamb Ltd. Manufacturer's address: Units 4 & 5, Parkview industrial estate, Eastbourne, East Sussex, BN23 6QE England.

METHOD:

1. Carefully fill the water bath with distilled water ensuring the electrical connection points remain dry.
2. Turn on water bath from the plug and set the power switch from O to I
3. The water will need to be heated to 40°C. The best way do to this is to first turn the temperature dial to 10 (maximum setting) for 7-8 minutes and then turn the dial down to 2.5. If the temperature of the bath is above 40°C, pour in distilled water to lower the temperature.
4. Scrape away any surplus wax from the edges of the cassette to ensure a good fit in the holder then place the tissue block in the refrigerator. Cool the block for at least 30 min prior to cutting.
5. Ensure that the handwheel is locked in the upper range of the vertical movement by turning the lever downwards.
6. Press the reverse course feed button  ove the knife carrier away from the specimen.

7. Turn the clamping lever on the blade holder to the front and swing the protective bracket forwards. Insert the feather S35 blade into the slot behind the clamping plate ensuring the blade is level on the rail (use forceps to manipulate the blade if necessary). Use half of the blade for cutting at a time. Preferably use left side first and then the right side.
8. Return the clamping lever to the original position (in line with the black frame of the blade) to lock the blade in place. The protective bracket should also be returned to the original position when the microtome is not in use to minimise the risk of sharps injury.
9. The blade angle is pre-set to 10-12° and should not require adjustment. These settings should remain the same to ensure minimal amounts of tissue are wasted when different laboratory workers prepare sections from the same block.
10. Insert the specimen against the 'fixed jaw' of the universal cassette clamp and secure by pulling the lever to the front. Ensure that each specimen cassette is always inserted in the same orientation (horizontal placement in the universal cassette clamp with the specimen number on the left) and inserted to the far left of the clamp.
11. Unlock the handwheel by turning the lever upwards. Rotate handwheel in a clockwise direction until the centre of the tissue block is level with the blade holder. Then lock the handwheel by turning the lever downwards.
12. Press the forward course feed button  ove the blade carrier near to the specimen. To establish whether the blade is close to the specimen view the distance between the two at a side angle. Also, gently move the handwheel up and down to see if the specimen is not touching the blade. When you are satisfied that both blade and specimen are close to each other move the handwheel so the specimen is away from the blade, this prevents scoring the specimen block in the middle.
13. The required section and trimming thickness are set by means of a circular knob on the left of the instrument. Press the circular control knob to switch between section (FEED) and trimming (TRIM) thickness. The corresponding LED will be displayed on the operating control panel:
    - Green LED lights up when the FEED function is used
    - Yellow LED lights up when the TRIM function is selected.

14. When a new tissue block is used, select TRIM and turn the control knob until the corresponding LED is set at 20µm.
15. Unlock the handwheel and start trimming by rotating the handwheel in a clockwise direction. Once you begin to see specks on tissue in the sections change the TRIM settings from 20µm to 10µm.
16. Continue to trim on the new setting until a representative amount of tissue is exposed in the tissue block.
17. Select FEED and turn the control knob until the corresponding LED is set at the required section thickness (usually 3-5µm). Rotate the handwheel until the first section generated. Gently hold the end of the section with forceps and continue to cut until a ribbon (consisting of approximately 6 sections) is produced. Discard the first few sections when first cutting on the feed section as usually the first few are too thick.
18. Carefully float the sections onto the pre-warmed water bath. Leave or a minute or so until the wrinkles in the section disappear. Separate the sections by applying gentle pressure using forceps. Select the best sections and float onto APES-coated slides. It is considered to be good laboratory practice to attach each section in the same orientation on the surface of the slide where you can read the IVD sign.
19. Label the frosted part of the slide with the specimen ID and thickness (using a pencil). Place slides in a rack to dry at room temperature for several hours (preferably over night).
20. When not cutting for a few minutes the specimen block moves slightly out of place which potentially can create thicker sectioning when you go back to cutting. To avoid creating thicker sections press the reverse course feed but  very slightly to move the knife carrier away from the specimen by a small amount.
21. During the cutting procedure a build up of wax on either side of the blade carrier. This can potentially cause scoring of the section. Using a soft brush, brush in an upward direction against the blade carrier to remove the wax, this is to prevent blunting of the blade and cutting down of the brush if a downward direction was used.
22. If the sections start to wrinkle during the cutting procedure, then the tissue block has become too warm and needs to be refrigerated for at least 10 minutes. To

remove the tissue block, press the reverse course feed button until the specimen is safely away from the blade holder. Return the safety bracket to the upright position. Remove the tissue block by pressing the lever on the universal cassette clamp.

23. Remove any unused tissue sections from the water bath surface using kitchen roll.
24. When the microtome blade needs to be replaced, return the tissue block to the furthest position using the course feed button and lock the handwheel. Unlock the lever on the blade carrier and place the microtome blade into the yellow clinical waste sharp bin. NEVER try to remove the microtome blade when the tissue block is close to the blade edge because there is a risk of sharps injury and also the risk of damaging the tissue block.
25. When the sections have dried, place the slides into a staining rack and bake for 60 minutes in a slide drier (pre-warmed to 60°C), or 37º C overnight. Place slides in a suitable 'dust free' container until required for the immunohistochemical staining procedure.

7.3.f ANTIGEN RETRIVAL

BACKGROUND

The mechanisms of formalin-fixation have been thought to be due to the formation of cross-linking bonds between tissue proteins, stabilising them to withstand subsequent processing (Mason and O'Leary 1991). The formation of cross linking bonds may, however mask the antigenic sites, thereby producing weak or false negative staining during immunohistochemical detection of certain proteins. The citrate based solution used in this procedure is designed to break the protein cross-links, therefore unmasking the antigens and epitopes in formalin-fixed and paraffin embedded tissue sections, thus enhancing subsequent staining intensity of antibodies.

HEALTH AND SAFETY REQUIREMENTS

Care needs to be taken when performing this procedure because there is a risk burns from the hotplate and pressure cooker. There is also a risk of chemical burns from sodium hydroxide and there is a mild irritant effect from citric acid. Suitable personal protection equipment (Nitrile gloves, safety glasses and a lab coat etc) should be worn. Refer to the physical hazards safety circular, risk assessments and COSHH forms before starting procedure. DO NOT COMMENCE WORK WITH THE PRESSURE COOKER UNTIL SUFFICIENT TRAINING HAS BEEN PROVIDED AND RECEIVED AUTHORISATION FROM LISA HEATHCOTE OR JO DRURY. THE SEAL ON THE PRESSURE COOKER SHOULD BE INSPECTED PRIOR TO EVERY USE AND REPLACED ANNUALLY.

EQUIPMENT INFORMATION:

- Sodium Hydroxide pellets and citric acid are supplied by VWR International Ltd, Poole, BH15 1TD England (Future orders will be obtained from Sigma or Thermo Fisher).
- Tefal Clipso Easy 6L pressure cooker and Russell Hobbs hotplate are obtained commercially from John Lewis department store. Annual Insurance check TBC. The seal/gasket needs to be replaced annually. Gasket (part number SA793145) is obtained directly from: http://www.homeandcook.co.uk refer to section accessories/pressure cookers. 2010 price £8.50 +£1.50 delivery
- Slide racks are supplied by Raymond A Lamb Ltd. Manufacturer's address: Units 4 & 5, Parkview industrial estate, Eastbourne, East Sussex, BN23 6QE England.

METHOD:

- Prepare a 10mM solution of citrate buffer, pH=6.0 by adding 3.15g citric acid to 1.5 litre of distilled water.
- Adjust pH to 6.0 with 2M NaOH.
- Remove covering lids off hotplate and turn on the hotplate to "6".
- Place citrate buffer in the pressure cooker and place lid back on pressure cooker. Bring the buffer to a rolling boil.

- Immerse the slide rack in the buffer using long forceps.
- Engage the lid, turn the valve to pressure symbol and press the lid down until it clicks.
- Set timer for 1 minute, when steam starts to vent, start timer. After 1 minute, turn off the hotplate and release the pressure by turning the vent to the "steam" symbol, and cool as quickly as possible by transferring the pressure cooker to the sink and running cold water.
- Transfer the slides back to the glass staining jar containing distilled water.
- Transfer to TBS ensuring that the tissue does not dry out.

7.3.g IMMUNOHISTOCHEMISTRY

Background

Immunohistochemical staining is a valuable tool for detecting specific antigens in tissues. In order to perform the standard staining procedure, first the tissue section has to be prepared (SOP 04, 05 and 06), deparaffinized and then rehydrated (SOP 08). Antigen retrieval (SOP 09) is then performed to break any protein cross-links, therefore unmasking the antigens and epitopes in the tissue sections. The DakoCytomatation EnVision+ System, HRP is a two-step Immunohistochemical staining technique. This system is based on an HRP labelled polymer which is conjugated with secondary antibodies. The labelled polymer does not contain avidin or biotin. Consequentially non-specific staining resulting from endogenous avidin-biotin activity in liver, kidney, lymphoid tissues and cryostat sections is eliminated or significantly reduced. The interpretation of any positive staining or its absence should be complemented by morphological and histological studies with proper counts. Any endogenous peroxidase activity is quenched by incubating the specimen for five minutes with Peroxidase block. The specimen is incubated with an appropriately characterised and diluted mouse primary antibody, followed by incubation with the labelled polymer, using two sequential 30-minute incubations. Staining is completed by 10 minute incubation with 3'3-Diaminobenzindine (DAB)+ substrate - chromogen which results in a brown – coloured precipitate.

Definition

This SOP will explain how to stain endometrial cells using the anti CD56 antibody. Please read entire procedure before staining sections. Perform all antibody and staining incubations in a humid chamber and do not allow sections to dry out. Isotype and system controls should also be run. Carefully time all tests. Do not touch test specimens on slides during the staining procedure, and ensure the slides do not dry out during the staining protocol.

Health and Safety Precautions

The main health and safety risks to this procedure arise from chemicals/reagents that are potentially hazardous, such as hydrogen peroxide (H_2O_2), xylene, ethanol, and DAB (3'3-Diaminobenzindine). Gloves must always be worn when working with any of these chemicals.

EQUIPMENT INFORMATION

Staining dishes

Humidified chamber

Cover slips

General points:

Ensure that solutions cover the whole specimen – if necessary use a piece of Parafilm to spread.

Antibody information:

Antigen	Antibody /cat#	Company	Dilution	duration
CD56	NCL-L-CD56-1B6	Novocastra	1:100	60 mins
Mouse IgG Negative Control	MCA928	Serotech	1:100	60 mins

METHOD (Paraffin sections):

1. Place slides in a staining dish containing TBS and incubate 5 minutes at room temperature.
2. Prepare 0.3% H_2O_2/TBS (2.5 ml 30% H_2O_2 + 247.5 ml TBS)

3. Incubate slides in 0.3% H_2O_2/TBS bath 10 minutes at room temperature.
4. Prepare humidified chamber by placing folded paper towels in the centre gulley's and soaking with distilled water.
5. Tap off any remaining solution, carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.
6. Place slides in a staining dish filled with TBS and incubate 5 minutes at room temperature.
7. Tap off any remaining solution, carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.
8. Mark area to be stained with DAKO hydrophobic marker pen ensuring that the tissue is surrounded with sufficient space to allow spreading of antibodies.
9. Place slides in a staining dish filled with TBS and incubate 5 minutes at room temperature.
10. Prepare antibody diluent (TBS/0.5 % BSA eg. 250 µl 10% BSA + 4750 µl TBS)
11. Prepare 1:100 dilutions of each antibody, allowing 50 µl per section.
12. Tap off any remaining solution, carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.
13. Place in humidified chamber.
14. Apply 50µl of the appropriate antibody to each section, spread with parafilm to ensure that the entire section is covered and incubate 60 min room temperature.
15. Tap off the antibody solution onto paper towels prior to placing the slides in a staining dish filled with TBS.
16. Incubate 5 minutes at room temperature.
17. Decant TBS and refill.
18. Incubate 5 minutes at room temperature.
19. Tap off any remaining TBS, carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.
20. Return slides to the humidified chamber and apply 1 drop labelled polymer-HRP.
21. Spread with parafilm to ensure that the entire section is covered and incubate 30 min room temperature.

22. Tap off the polymer solution onto paper towels prior to placing the slides in a staining dish filled with TBS.
23. Incubate 5 minutes at room temperature.
24. Decant TBS and refill.
25. Incubate 5 minutes at room temperature.
26. Prepare substrate/chromagen solution: 20µl/1ml substrate (require 50 µl per section).
27. Tap off any remaining TBS, carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.
28. Return slides to the humidified chamber and apply 50 µl substrate/chromagen solution.
29. Spread with parafilm to ensure that the entire section is covered and incubate 10 min room temperature.
30. Place slides in staining rack and immerse immediately in tap water to stop the reaction.
31. Turn on fume hood in lab 4.
32. Counterstain using filtered Gill 2 haematoxylin in lab 4. Immerse for 1 min 30s.
33. Immerse in tap water and rinse until water is clear.
34. Dip briefly in acid alcohol, and immediately back into tap water (5 min).
35. Incubate 1 min in 70% ethanol.
36. Blot off excess and incubate 1 min in 90% ethanol.
37. Blot off excess and incubate 3 min in 100% ethanol.
38. Repeat step 36.
39. Blot off excess and incubate 5 min in xylene.
40. Blot off excess and incubate 10 min in xylene.
41. Remove a few slides at a time and apply sufficient DPX to cover the section.
42. Choose an appropriately sized coverslip and apply to the slide.
43. Remove air bubbles by using a cocktail stick or yellow pipette tip to gently press on the coverslip and “chase” bubbles to the edge.
44. Leave to dry in fume hood.